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(57) Abstract

Isolated polypeptides useful in ameliorating GAD-associated autoimmune disease as well as diagnostic and therapeutic methods of using the peptides are disclosed.

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CLONED GLUTAMIC ACID DECARBOXYLASE

This application is a continuation-in-part application of U.S. Serial No. 07/716,909, filed June 18, 1991 which is a continuation-in-part application of U.S. Serial No. 07/586,536, filed September 21, 1990.

BACKGROUND OF THE INVENTION

The present invention was supported by Grant NS22256 from the National Institutes of Health. The United States Government has certain rights in this invention.

1. FIELD OF THE INVENTION

The present invention relates to glutamic acid decarboxylase$_{65}$ (GAD_{65}) polypeptides and methods of using GAD_{65} polypeptides diagnostically and therapeutically in autoimmune disease.

2. DESCRIPTION OF THE BACKGROUND ART

Insulin-dependent diabetes mellitus (IDDM; type I diabetes) is one of the most common metabolic disorders. In the United States, IDDM affects approximately one in 300 to 400 people, and epidemiological studies suggest that its incidence is increasing. The disease results from the autoimmune destruction of the insulin-producing β-cells of the pancreas. More specifically, the preonset stage is characterized by "insulitis", in which lymphocytes infiltrate the pancreatic islets and selectively destroy the β-cells. Insulitis may be present for many years before the onset of clinical symptoms. The typical IDDM presentation of hyperglycemia appears only after at least 80% of the insulin-producing β-cells are lost. The remaining β-cells are destroyed during the next few years.

Although insulin therapy allows most IDDM patients to lead normal lives, this replacement is imperfect and does not completely restore metabolic homeostasis. Thus, severe complications which result in dysfunctions

of the eye, kidney, heart, and other organs are common in IDDM patients undergoing insulin therapy. Because of this, it is highly desirable to extend the latency period and prevent progression (e.g., through administration of immunosuppressant drugs to interfere with the autoimmune process and insulin to achieve better control of the effects of sustained hypoglycemia) between the start of β-cell destruction and the actual requirement of insulin replacement (i.e., when 80% of the β-cells are destroyed). Therefore, a diagnostic test which determines the beginning of β-cell destruction would allow the clinician to administer immunosuppressant drugs (Silverstein, *et al., New England Journal of Medicine*, 319:599-604, 1988) or prophylactic insulin therapy (Keller, *et al., Lancet*, 341:927, 1993) to extend this latency period and thus significantly delay the onset of insulin replacement side effects.

Many IDDM patients have sera which contain antibodies to a 64kD molecule (Baekkeskov, *et al., J.Clin.Invest.*, 79:926-934, 1987; Atkinson, *et al., Lancet*, 335:1357-1360, 1990), to islet cell cytoplasmic (ICA) molecules or islet cell surface (ICSA) molecules (Bottazzo, *et al, Lancet*, 1:668-672, 1980), or to insulin (Palmer, *et al., Science*, 222:1137-1139, 1983; Atkinson, *et al., Diabetes*, 35:894-898, 1986). Atkinson and coworkers (Atkinson, *et al., Lancet*, 335:1357-1360, 1990) have demonstrated that the presence of antibodies to the 64kD molecule in human sera appears to be the earliest and most reliable indicator that onset of IDDM symptoms will eventually occur.

Recently, Baekkeskov and coworkers established that the 64kD molecule and glutamic acid decarboxylase (GAD) have several antigenic epitopes in common and thus they may be identical or very similar molecul s. Although this identification is an important finding, the use of

this information as a diagnostic tool for predicting IDDM is quite cumbersome and limited unless knowledge of the molecular biology of GAD is known. Studies by Kaufman, *et al.*, (*J. Clin. Invest.*, 89:283, 1992) established that the 64kD molecule was intact GAD_{65}. Consequently, the cloning and subsequent production of large quantities of GAD_{65} or a GAD molecule which is antigenically substantially identical to the GAD_{65} molecule or fragments of the GAD_{65} molecule, both of which can be easily purified, will allow the development of a diagnostic kit designed to predict IDDM as well as effective therapeutic modalities. The present invention provides a means for accomplishing these results.

SUMMARY OF THE INVENTION

The present invention arose out of the discovery that recombinant DNA technology could be used to produce eukaryotic GAD_{65} polypeptide and that GAD_{65} polypeptide could be used in the diagnosis and therapy of patients with autoimmune disease. Particularly relevant is the use of eukaryotic GAD_{65} polypeptide in the diagnosis and therapy of patients having, or at risk of having, GAD-associated autoimmune disorders such as insulin-dependent diabetes mellitus (IDDM) or stiff man disease.

A major advantage of the present invention is that it provides the art with a ready source of eukaryotic GAD_{65} polypeptide corresponding to that purified from natural sources, while avoiding the problems associated with the isolation of naturally occurring eukaryotic GAD_{65} polypeptide when separating it from other eukaryotic non-GAD_{65} polypeptides. This absence of other eukaryotic non-GAD_{65} polypeptides is significant in that it allows the development of test systems which will only detect antibodies specifically reactiv with GAD_{65} polyp ptides.

Another advantage of providing eukaryotic GAD_{65} polypeptide in host cells is that by so doing, it is possible to obtain much larger quantities of the polypeptide than are currently practicably available from natural sources. As a consequence, not only is it possible to use the polypeptide of the invention to more accurately classify and treat patients with such autoimmune diseases as IDDM, but it is also now possible to provide commercially useful quantities of GAD polypeptide for use in diagnostic systems and pharmaceutical compositions.

DESCRIPTION OF THE DRAWINGS

FIGURE 1 Cloning strategy for obtaining GAD_{65} and GAD_{67} specific cDNA probes.

FIGURE 2 DNA sequence and corresponding amino acid sequence for rat GAD_{65}.

FIGURE 3 DNA sequence and corresponding amino acid sequence for human GAD_{65}.

FIGURE 4 Comparison of rat GAD_{65} and human GAD_{65} amino acid sequences.

FIGURE 5 GAD_{65} and GAD_{67} cDNAs hybridize to different size RNAs.

FIGURE 6 Southern blots hybridized with CDNA probes specific for GAD_{65} and GAD_{67}.

FIGURE 7 Immunological identification of GAD_{65} and GAD_{67}.

FIGURE 8 Proliferative T-cell responses of NOD mice to β cell antigens.

FIGURE 9 Characterization of GAD specific T-cell response of NOD mice as primed Th1 cells by enhanced clonal size (a) and cell surface markers (b) and IFNγ secretion.

FIGURE 10 Intramolecular spreading of T-cell autoimmunity within the GAD molecule.

FIGURE 11 Delay of onset of IDDM following immunization with GAD_{65}.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the manipulation of genetic materials by recombinant DNA procedures which make possible the production of polypeptides possessing part or all of the primary structural conformation for one or more of the epitopes for binding autoantibodies to glutamic acid decarboxylase$_{65}$ (GAD_{65}) and for polypeptides that bind to MHC receptors to block T-cell recognition. These polypeptides are highly useful for the immunological detection of autoantibodies reactive with them, since such autoantibodies are pre-diagnostic and indicative of autoimmune diseases such as insulin dependent diabetes mellitus and "stiff man" syndrome. These polypeptides can also be used for purposes of screening drugs, such as those that alter GAD function, and for generation of polyclonal and monoclonal antibodies which, in turn, can be used diagnostically to detect GAD_{65}.

The development of specific DNA sequences encoding eukaryotic GAD_{65} polypeptide for splicing into DNA vectors can be accomplished using a variety of techniques. For example, alternative methods which can be employed include (1) the isolation of a double stranded DNA sequence from the genomic DNA of the eukaryote; (2) the chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest; and (3) the *in vitro* synthesis of a double stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double stranded DNA complement of MRNA is eventually formed which is generally referred to as CDNA.

The manufacture of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is known. When th ntire sequence f amino acid residues of th d sired polypeptide is not known, the direct manufacture of DNA sequences is not possible and the method f choic is the formation of cDNA sequences.

Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid-carrying cDNA libraries which are derived from reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single stranded form (Jay, *et al.*, *Nucleic Acid Research*, 11:2325, 1983).

Hybridization procedures are useful for the screening of recombinant clones by using labeled mixed synthetic oligonucleotide probes wherein each is potentially the complete complement of a specific DNA sequence in the hybridization sample which includes a heterogeneous mixture of denatured double stranded DNA. For such screening, hybridization is preferably performed on either single stranded DNA or denatured double stranded DNA. These procedures are particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. In other words, by using stringent hybridization conditions directed toward avoidance of non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixtur which is its complet complement (Wallace, *et al.*, *Nucleic Acid Research*, 9:879, 1981).

In addition, a GAD cDNA library can be scre ned by injecting the various cDNAs into oocytes, allowing sufficient tim for xpression f th cDNA gene products to occur, and testing for the presence of the

desired cDNA expression product, for example, by using antibody specific for GAD_{65} polypeptide, by using functional assays for GAD_{65} enzymatic activity, or by measuring the ability of the expression product to stimulate pathogenic T-cells.

Alternatively, a cDNA library can be screened indirectly for GAD_{65} peptides having at least one epitope using antibodies to GAD_{65} (Chang and Gottlieb, *J.Neurosci.*, 8:2123, 1988). Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of GAD_{65} cDNA. Preferred are antibodies directed to an epitope found in the first 100 amino acids of the N-terminal portion of GAD_{65}.

Of the three above-noted methods for developing specific DNA sequences for use in recombinant procedures, the use of genomic DNA isolates, is the least common. This is especially true when it is desirable to obtain the microbial expression of mammalian polypeptides because of the presence of introns.

The present invention provides novel polypeptides of GAD_{65} which have part or all of the primary structural conformation, that is, a continuous sequence of amino acid residues, having at least one epitope for antibodies to GAD_{65} or at least one determinant for T-cell recognition. It is possible to use the polypeptide fragments of the invention rather than intact GAD to detect autoantibodies to GAD. The term "polypeptide," as applied to GAD polypeptide, includes any sequence of amino acids having an epitope for autoantibodies to GAD or binds to a T-cell MHC receptor. Thus, the polypeptide fragments of GAD encompassed by the invention possess a biological activity such as the ability to induce and/or bind autoantibodies to GAD, bind to T-cell MHC receptors (especially receptors on pathogenic T-cells) and the like.

The polypeptides resulting from microbial expression of the DNA sequenc s of the invention or from other synthetic techniques, such as solid-phase peptide synthesis, can be further characterized by their freedom from association with other eukaryotic polypeptides or other contaminants which might otherwise be associated with GAD in its natural cellular environment or in such extracellular fluids as plasma or urine.

Studies by the present inventors unequivocally establish that GAD_{65} and GAD_{67} are encoded by distinct genes and are not produced, for example, by post-transcriptional or post-translational modification of a common genomic sequence. Evidence proving that GAD_{65} and GAD_{67} are encoded by different genes include: (a) the largest contiguous sequence of exact identity between GAD_{65} and GAD_{67} cDNAs is only 17 nucleotides in length, (b) cDNAs from GAD_{65} and GAD_{67} do not cross hybridize with each other's or with each other's mRNA under low stringency conditions (2.0 x SSC, 0.01% SDS, 23°C), and (c) GAD_{65} and GAD_{67} cDNAs do not cross hybridize with isolated genomic clones encoding GAD_{67} and GAD_{65}, respectively.

The term "host" includes not only prokaryotes, but also such eukaryotes as yeast, filamentous fungi, plant and animal cells, as well as insect cells which can replicate and express an intron-free DNA sequence of eukaryotic GAD_{65}. However, prokaryotes are preferred as the host organism for screening purposes while eukaryotic cells, especially insect cells, are preferred for expression.

The term "prokaryotes" includes all bacteria which can be transformed or transfected with the gene for the xpression of GAD_{65}. Prokaryotic hosts may include gram negative as well as gram positive bacteria such as, for example, *E. coli, S. typhimurium, Serratia marc scens and Bacillus subtilis*.

A recombinant DNA molecule coding for the GAD_{65} polypeptides can be used to transform or transfect the host using any of the techniques commonly known to those of ordinary skill in the art. Especially preferred is the use of a plasmid or a virus containing the GAD_{65} coding sequence for purposes of prokaryotic transformation or transfection, respectively. Alternatively, liposomes containing the DNA of interest can be used to obtain expression in the host (Zhu, *et al.*, *Science*, 261:209, 1993)

Methods for preparing fused, operably linked genes and expressing them in bacteria are well-known in the art (Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). The genetic constructs and methods described therein can be utilized for expression of GAD_{65} in prokaryotic hosts.

In general, expression vectors containing promotor sequences which facilitate the efficient transcription of the inserted eukaryotic genetic sequence are used in connection with the host. The expression vector typically contains an origin of replication, a promoter, and a terminator, as well as specific genes which are capable of providing phenotypic selection of the transformed cells. The transformed prokaryotic hosts can be grown in fermentors and cultured according to techniques known in the art to achieve optimal cell growth. The polypeptides of the invention can then be isolated from the grown medium, cellular lysates, or cellular membrane fractions.

The isolation and purification of the expressed polypeptides of the invention may be by any conventional means such as, for example, preparative chr mat graphic separations and immunological separations such as those involving the use of m n clonal or polyclonal antibody.

By having provided the sequence of amino acid residues of GAD_{65}, the present invention provides for the manufacture of DNA sequences which code for the

host expression of polypeptide analogs or derivatives of GAD_{65} which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues and which share some or all of the epitopes of naturally-occurring polypeptide forms.

The novel DNA sequences of the invention include all sequences useful in providing the expression in prokaryotic or eukaryotic host cells of polypeptides which have at least a part of the primary structural conformation for one or more epitopes capable of reacting with autoantibodies to GAD_{65} which are comprehended by: (a) the DNA sequence as set forth in Figures 2 or 3 or their complementary strands; (b) DNA sequences which hybridize to DNA sequences defined in (a) or fragments thereof; and (c) DNA sequences which, but for the degeneracy of the genetic code, would hybridize to DNA sequences defined in (a) and (b) above. Specifically comprehended in (b) are genomic DNA sequences which encode allelic variant forms of GAD_{65}. Part (c) specifically comprehends the manufacture of DNA sequences which encode GAD_{65}, GAD_{65} fragments, and GAD_{65} analogs wherein the DNA sequences thereof may incorporate codons which facilitate translation of mRNA in non-vertebrate hosts.

Since the cDNA sequence of the invention encodes essentially the entire human or rat GAD_{65} molecule, it is now a matter of routine to prepare, subclone, and express smaller polypeptide fragments of cDNA from this or a corresponding cDNA sequence which would encode as few as one epitope for autoantibodies to human or rat GAD_{65}. The presence of such an epitope on a cloned polypeptide can then be confirmed using, for example, serum from a patient with autoantibodies to GAD_{65}. An exampl of such a smaller peptide is the first approximately 100 amino acids from the N-terminus of GAD_{65} (shown in Figure 3). This amino acid sequence is essentially absent from GAD_{67}. Other examples of specific peptides of the invention are shown in Table 7

as well as the approximate carboxy-terminal two-thirds of GAD from about amino acid 224 to about amino acid 585. Especially preferred in the carboxy-terminal two-thirds of GAD is the amino acid segment from about amino acid 224 to about amino acid 398.

The present invention further relates to monoclonal antibodies which are specific for the polypeptides of the invention as well as the diagnostic and therapeutic use of these monoclonal antibodies. This specificity enables the monoclonal antibody, and like monoclonal antibodies with like specificity, to be used to bind the polypeptide of the invention when the polypeptide, or amino acids comprising the polypeptide, are present in specimens or a host, such as a human.

Numerous techniques can be utilized to produce the monoclonal antibodies of the invention without resorting to undue experimentation. To a great extent, the products of such monoclonal antibodies is rendered routine because of the highly defined nature of the polypeptides of the invention. Thus, whether the polypeptides of the invention are used for immunization and/or screening, the very limited number of immunogenic determinants on the polypeptides greatly simplifies the identification of cell lines producing monoclonal antibodies of the invention, for example, by limiting the repertoire of clonal expression possible.

One very useful type of cell line for expression of the monoclonal antibodies of the invention is the hybridoma. The general method used for production of hybridomas producing monoclonal antibody is well known (Kohler and Milstein, *Nature*, 256:495, 1975). The resulting hybridomas were then screened for production of monoclonal antibodies capable of binding to the polypeptides of the invention.

The techniques of sensitization and/or immunization, cell fusion, ascites production, selecti n of mixed hybridomas, or subcloning of monoclonal hybridomas are generally well known in the art. Attention is direct d to Koprowski, *et al.*, U.S.

Patent No. 4,172,124, Koprowski, *et al.*, U.S. Patent No. 4,196,265, or Douillard, J.Y. and Hoffman, T., *Basic Facts about Hybridomas*, in *Compendium of Immunology*, Vol. II, L. Schwartz, ed. (1981), which are herein incorporated by reference. In general, the purified peptides can be modified to have a cystine attached at the C-terminus to permit unidirectional attachment of the synthetic peptide to an immunogenic protein through a connecting bridge, for example, maleimidobenzoylated (MB)-keyhole limpet hemocyanin (KLH). Other immunogenic conjugates can also be used, for example, albumin, and the like. The resulting structure may have several peptide structures linked to one molecule of protein.

Somatic cells derived from a host immunized against the synthetic peptides can be obtained by any suitable immunization technique. The host subject is immunized by administering the antigen, usually in the form of a protein conjugate, as indicated above, by any suitable method, preferably by injection, either intraperitoneally, intravenously, subcutaneously, or by intra-foot pad. Adjuvants may be included in the immunization protocol.

The initial immunization with the protein bound antigen can be followed by several booster injections given periodically at intervals of several weeks. The antibody contained in the plasma of each host can then be tested for its reactivity with the immunizing polypeptide of the invention. The host having the highest response is usually most desirable as the donor of the antibody secreting somatic cells used in the production of hybridomas. Alternatively, hyperimmunization can be effected by repeatedly injecting additional amounts of peptide-protein conjugate by intravenous and/or intraperiton al route.

The isolation of hybridomas producing monoclonal antibodies of the invention can be accomplished using routine screening techniques which permit determination of the elementary reaction pattern of the monoclonal antibody of interest. Thus, if a monoclonal antibody being tested binds with a polypeptide of the invention, then the antibody being tested and the antibody produced by the hybridomas of the invention are equivalent.

Alternatively, since the invention teaches polypeptides or amino acid sequences which are specifically required for binding of the preferred monoclonal antibodies of the invention, it is now possible to use these peptides for purposes of immunization to produce hybridomas which, in turn, produce monoclonal antibodies specific for the polypeptide. This approach has the added advantage of decreasing the repertoire of monoclonal antibodies generated by limiting the number of antigenic determinants presented at immunization by the polypeptide. The monoclonal antibodies produced by this method can be screened for specificity using standard techniques, for example, by binding polypeptide to a microtiter plate and measuring binding of the monoclonal antibody by an ELISA assay.

It is also possible to determine, without undue experimentation, if a monoclonal antibody has the same specificity as a monoclonal antibody of the invention by ascertaining whether the former prevents the latter from binding the polypeptide of the invention. If the monoclonal antibody being tested competes with the monoclonal antibody of the invention, as shown by a decrease in binding by the monoclonal antibody of the invention, then it is likely that the two monoclonal antibodies bind to the same, or a closely related, epitope.

Still another way to determine whether a monoclonal antibody has the sp cificity of a monoclonal antibody of the invention is to pr -incubate the monoclonal

antibody of the invention with the polypeptide of the invention with which it is normally reactive, and then add the monoclonal antibody being tested to determine if the monoclonal antibody being tested is inhibited in its ability to bind the antigen. If the monoclonal antibody being tested is inhibited then, in all likelihood, it has the same, or a closely related, epitopic specificity as the monoclonal antibody of the invention.

The GAD_{65} of the invention is particularly suited for use in immunoassays in which it can be utilized in liquid phase or bound to a solid phase carrier. In addition, GAD_{65} used in these assays can be detectably labeled in various ways.

Examples of immunoassays which can utilize the GAD_{65} of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such immunoassays are the radioimmunoassay (RIA), the sandwich (immunometric assay) and the Western blot assay. Detection of antibodies which bind to the GAD_{65} of the invention can be done utilizing immunoassays which run in either the forward, reverse, or simultaneous modes, including immunohistochemical assays on physiological samples. The concentration of GAD_{65} which is used will vary depending on the type of immunoassay and nature of the detectable label which is used. However, regardless of the type of immunoassay which is used, the concentration of GAD_{65} utilized can be readily determined by one of ordinary skill in the art using routine experimentation.

The GAD and GAD fragments of the invention can be bound to many different carriers and used to detect the presence of antibody specifically reactive with the polyp ptide. Alternatively, the carrier-bound GAD and GAD fragments can be used therapeutically for extracorporeal absorption of autoimmune antibodies in patients having, r at risk of having, GAD-associated disorders. Examples of well-known carriers include glass, polystyren , polyvinyl chloride, polypropylene,

polyethylene, p lycarbonate, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses, and magnetite. The nature of the carrier can be either soluble or insoluble for purposes of the invention. Those skilled in the art will know of other suitable carriers for binding GAD_{65}, or will be able to ascertain such, using routine experimentation.

There are many different labels and methods of labeling known to those of ordinary skill in the art. Examples of the types of labels which can be used in the present invention include enzymes, radioisotopes, colloidal metals, fluorescent compounds, chemiluminescent compounds, and bioluminescent compounds.

Alternatively, the polypeptide of the invention which comprises the GAD enzymatic domain can be used to detect antibodies to GAD by measuring GAD enzymatic activity. For example, GAD_{65} and a specimen suspected of having antibodies to GAD_{65} can be incubated for a period of time and under conditions sufficient to allow binding to occur between GAD_{65} and the antibodies. The reaction product is precipitated and then tested for GAD enzymatic activity.

For purposes of the invention, the antibody which binds to GAD_{65} of the invention may be present in various biological fluids and tissues. Any sample containing a detectable amount of antibodies to GAD_{65} can be used. Normally, a sample is a liquid such as urine, saliva, cerebrospinal fluid, blood, serum and the like, or a solid or semi-solid such as tissue, feces and the like.

The materials for use in the assay of the invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentaliz d to receive in close confinement one or mor container m ans such as vials, tubes and the like, ach of the container means comprising one of the separat lements to be used in the method. For example, one of the container means may comprise GAD_{65}

bound to a carrier. A second container may comprise soluble, detectably-labeled second antibody, in lyophilized form or in solution.

In addition, the carrier means may also contain a plurality of containers each of which comprises different, predetermined amounts of GAD_{65}. These latter containers can then be used to prepare a standard curve into which can be interpolated the results obtained from the sample containing the unknown amount of autoantibodies to GAD_{65}.

In using the kit all the user has to do is add, to a container, a premeasured amount of a sample containing a measurable, yet unknown amount of autoantibodies to GAD_{65} to be detected, a premeasured amount of carrier-bound GAD_{65} present in the first container, and a premeasured amount of the detectably labeled second antibody present in the second container. Alternatively, the non-detectably labeled GAD_{65} can be provided attached to the container to which the sample and the detectably labeled second antibody are added. After an appropriate time for incubation, an immune complex is formed and is separated from the supernatant fluid, and the immune complex or the supernatant fluid are detected, as by radioactive counting or addition of an enzyme substrate, and color development.

In an alternative embodiment, a kit comprising the GAD polypeptide of the invention can be used to detect the stage of GAD-associated autoimmune disease in a patient. As further shown herein, Applicants have discovered that certain GAD peptides or fragments are associated with different levels of progression in the autoimmune disease and that the level of disease process can be ascertained by looking at immune cell proliferative response, such as that of the pathogenic T-c ll of the patient.

The term "ameliorate" denotes a lessening of the detrimental effect of the autoimmune response in the patient receiving therapy. The term "therapeutically

effective" means that the amount of GAD_{65} polypeptide used is of sufficient quantity to ameliorate the cause of disease due to the autoimmune response.

The GAD_{65} polypeptides, including whole GAD_{65}, of the invention can be used therapeutically in patients having, or at risk of having, an autoimmune response associated with GAD_{65}. Such therapy can be accomplished, for example, by the administration of GAD_{65} polypeptide to induce tolerance to GAD. Such administration can utilize unlabeled as well as labeled GAD_{65} polypeptide. When unlabeled GAD_{65} polypeptide is utilized advantageously, it would be in a form wherein, for example, the GAD_{65} polypeptides are in fragments which are too small to stimulate an immune response, but large enough to bind, or block, the continuance of the autoimmune response. For example, GAD_{65} could be digested enzymatically into epitope-sized peptides (typically 5-12 amino acids in length) and thereby bind to Fab binding portions present in the body fluids, or on the surface of immune cells, of the patient with autoimmune disease. Alternatively, peptides having at least one determinant for binding to T-cell MHC receptor can be similarly produced or chemically synthesized.

Alternatively, the GAD_{65} polypeptides of the invention can be administered labeled with a therapeutic agent. These agents can be coupled either directly or indirectly to the GAD_{65} polypeptides of the invention. One example of indirect coupling is by use of a spacer moiety. These spacer moieties, in turn, can be either insoluble or soluble (Diener, *et al.*, *Science*, 231:148, 1986) and can be selected to enable drug release from the GAD_{65} polypeptide at the target site. Exampl s of therapeutic agents which can be coupled to the GAD_{65} polypeptides of the invention for immunotherapy are drugs, radioisotopes, lectins, and toxins.

The drugs with which can be conjugated to the GAD_{65} polypeptides of the invention include compounds which are classically referred to as drugs such as mitomycin C, daunorubicin, and vinblastine.

In using radioisotopically conjugated GAD_{65} polypeptides of the invention for immunotherapy, certain isotopes may be more preferable than others depending on such factors as leukocyte distribution as well as stability and emission. Depending on the autoimmune response, some emitters may be preferable to others. In general, α and β particle-emitting radioisotopes are preferred in immunotherapy. Preferred are short range, high energy α emitters such as ^{212}Bi. Examples of radioisotopes which can be bound to the GAD_{65} polypeptides of the invention for therapeutic purposes are ^{125}I, ^{131}I, ^{90}Y, ^{67}Cu, ^{212}Bi, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd and ^{188}Re.

Lectins are proteins, usually isolated from plant material, which bind to specific sugar moieties. Many lectins are also able to agglutinate cells and stimulate lymphocytes. However, ricin is a toxic lectin which has been used immunotherapeutically. This is accomplished by binding the α-peptide chain of ricin, which is responsible for toxicity, to the antibody molecule to enable site specific delivery of the toxic effect.

Toxins are poisonous substances produced by plants, animals, or microorganisms that, in sufficient dose, are often lethal. Diphtheria toxin is a substance produced by *Corynebacterium diphtheria* which can be used therapeutically. This toxin consists of an α and β subunit which under proper conditions can be separated. The toxic A component can be bound to GAD_{65} polyp ptide and used for site specific delivery to a leukocyte expressing a receptor for GAD_{65} polypeptide.

Other therapeutic agents which can be c upled to the GAD_{65} polypeptides of the invention, as well as *ex vivo* and *in vivo* therapeutic protocols, are known, or

can be easily ascertained, by those of ordinary skill in the art.

The present invention also relates to a polypeptide which can be administered therapeutically to ameliorate, or utilized diagnostically to identify, the disease process in patients having, or at risk of having, this disease. The conventional single-letter code used to represent the various amino acids relates as follows:

TABLE 1

Phe: F	Leu: L	Ile: I	Met: M
Val: V	Ser: S	Pro: P	Thr: T
Ala: A	Tyr: Y	His: H	Gln: Q
Asn: N	Lys: K	Asp: D	Glu: E
Cys: C	Trp: W	Arg: R	Gly: G

A polypeptide sequence of the invention was identified by comparing the amino acid sequences of human GAD_{65}, human GAD_{67}, and the P2-C protein of the picornavirus, coxsackie virus. The P2-C polynucleotide plays a role in the virus membrane bound replication complex. These analyses established the presence of an extensive sequence similarity between both GAD_{65} molecules and the coxsackie virus. A core polypeptide of six contiguous amino acid residues of the GAD_{65} and P2-C polypeptide are identical in amino acid sequence. Indeed, of the 24 amino acids in the polypeptide, 19 are identical or conserved. In addition, there also exists a high charge density and the presence of a proline residue which would render this region highly antigenic (see Table 2).

TABLE 2

COMPARISON OF AMINO ACID SEQUENCES

Protein	Amino Acid Sequence
Human GAD_{67}	$_{258}$S I M A A R Y K Y F P E V K T K G M A A V P K L$_{281}$
Human GAD_{65}	$_{250}$A M M I A R F K M F P E V K E K G M A A L P R L$_{273}$
Coxsackie Virus P2-C	$_{28}$F I E W L K V K I L P E V K E K H E F - L S R L$_{50}$

The solid line encloses identical amino acids whereas the dashed line encloses amino acid residues with similar charge, polarity, or hydrophobicity.

In Table 2, the solid line ncloses identical amino acids whereas the dashed line encloses amino acid residues with similar charge, polarity, or hydrophobicity.

The discovery of this common polypeptide region supports an etiologic role for "molecular mimicry" in the precipitation of diabetes. Thus, where a patient genetically susceptible to IDDM is infected by a coxsackie virus, the immune response to the similar GAD sequence in the patient's β-cells. The immunological response is maintained by the antigenically similar GAD polypeptides resulting in the eventual destruction of the β-cells and the subsequent presentation of IDDM.

At present, it is believed that the destruction of pancreatic β-cells in IDDM is mediated by a cellular autoimmune response. As described herein, a polypeptide of the invention can ameliorate the autoimmune response to GAD. Because of the complexity of autoimmune disease, it is possible to envision numerous possible therapeutic modalities which would allow the polypeptides of the invention to be used to ameliorate such diseases. In one embodiment, it appears that the polypeptides of the invention can be utilized to block recognition by a specific T cell receptor (TCR) or an MHC receptor presenting an autoimmune antigen on the surface of an antigen presenting cell (APC). The inhibition of such recognition might occur, for example, by providing the patient with the polypeptide of the invention which, in turn, can displace the autoimmune antigen being presented in the antigen-cleft of the MHC receptor. However, although not wanting to be bound to a particular theory, it is believed that the polypeptides of the invention probably act to induce or restore a tolerogenic stat by direct interaction with the appropriat TCR on the surface of a GAD specific pathogenic T-cell. This latter therapeutic appr ach of

direct interaction with the TCR is supported by the examples and suggests that suppression of the autoimmune response can be achieved through induction of high-zone tolerance by use of high concentrations of polypeptide, preferably soluble. Another possible mechanism is that the polypeptide of the invention may play a role in anergizing pathogenic T cells by binding to the T cell MHC receptor, thereby preventing the appropriate costimulatory signal.

Alternatively, the polypeptides of the invention could be used to stimulate a T-suppressor cell population in order to restore self-recognition and, thereby, ameliorate the autoimmune disease. Stimulation of T-suppressor cell populations could be achieved, for example, by use of a bi-specific antibody having one variable region specific for an epitope present on the autoimmune antigen residing in the cleft of the MHCII receptor and, a second variable region specific for an epitope present on the $CD8^+$ receptor. The production of antibody specific for the polypeptide of the invention is a matter of routine to those of skill in the art, as is the preparation of bi-specific antibodies having specificity for 2 or more epitopes.

Polypeptide analogs of the present invention may be designed which will compete for recognition of self-antigens at the level of antigen presentation or induce anergy in T cells, due to a lack of a costimulatory signal. Since MHC molecules contain a single peptide binding site, it is possible to design polypeptides which will bind with high affinity to disease-associated MHC molecules, but will not activate disease-causing T-helper cells. Such polypeptides act as antagonists for self-antigen recognition. In the present invention, support for this mechanism is found in the examples, especially Example 7. Precedent for such an approach aris s from observation that a mouse lysozyme polypeptide, its lf non-immunogenic, can

compete for MHC binding with an immunogenic polypeptide from hen-egg white lysozyme and thereby reduce T cell activation by that polypeptide (Adorini, *et al.*, *Nature*, 334:623-625, 1988) as well as studies using T-cell receptor peptides to block formation of complex between T-cells, autoantigen and MHC (Howell, *et al.*, *Science*, 246:668, 1989). Similarly, such a therapeutic approach for screening effective polypeptide analogs has been utilized in such autoimmune diseases as experimental autoimmune encephalomyelitis (EAE) (Wraith, *et al.*, *Cell*, 59:248, 1989; Urban, *et al.*, *Cell*, 59:257, 1989).

The single-letter symbols used to represent the amino acid residues in the polypeptides of the present invention are those symbols commonly used in the art. The peptides of the invention include not only the natural amino acid sequences, but also peptides which are analogs, chemical derivatives, or salts thereof. The term "analog" or "conservative variation" refers to any polypeptide having a substantially identical amino acid sequence to a polypeptide provided herein and in which one or more amino acids have been substituted with chemically similar amino acids. For example, one polar amino acid, such as glycine or serine, may be substituted for another polar amino acid; or one acidic amino acid, such as aspartic acid may be substituted for another acidic amino acid, such as glutamic acid; or a basic amino acid, such as lysine, arginine, or histidine may be substituted for another basic amino acid; or a non-polar amino acid such as alanine, leucine, or isoleucine may be substituted for another non-polar amino acid.

The term "analog" or "conservative variation" also means any polypeptide which has one or more amino acids deleted from or added to a polypeptide of the present invention, but which still retains a substantial amino acid sequence homology to such peptide. A substantial

sequence homology is any homology greater than 70%, preferably at least about 80%, and more preferably at least about 90%. The term "fragment" also means any shorter version of the polypeptides identified herein having at least 6 amino acid residues, wherein the fragment possesses biological activity, or is a fragment capable of inhibiting the stimulation of T-cells by a stimulating polypeptide fragment or substantially full-length molecule.

The term "chemical derivative" means any polypeptide derived from a polypeptide of the present invention and in which one or more amino acids have been chemically derivatized by reaction of the functional side groups of amino acid residues present in the polypeptide. Thus, a "chemical derivative" is a polypeptide that is derived from the sequences or polypeptides identified herein by one or more chemical steps. Such derivatized molecules include, for example, those molecules in which free amino groups have been derivatized to form amine hydrochlorides, P-toluene sulfoamides, benzoxycarboamides, T-butyloxycarboamides, thiourethane-type derivatives, trifluoroacetylamides, chloroaceamides, or formamides. Free carboxyl groups may be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides. Free hydroxyl groups may be derivatized to form O-acyl or O-alkyl derivatives. The imidazole nitrogen of histidine may be derivatized to form N-im-benzylhistidine. Also included as chemical derivatives are those polypeptides which contain one or more naturally occurring amino acids derivatives of the 20 standard amino acids. For example, 4-hydroxyproline may be substituted for proline; 5-hydroxylysine may be substitut d for lysine; 3-methylhistidin may be substituted for histidine; homoserine may be substituted for serine, and ornithine may be substituted for lysine.

It should be understood that the present invention is not limited to the illustrative polypeptides depicted in Table 2 and Table 9, instead, a polypeptide falling within the scope of this invention may extend outside of, or comprise less than, the region between amino acid 28 and amino acid 50 of coxsackie virus P2-C, or between amino acid 250 and amino acid 273 of GAD_{65}, or between amino acid 258 and amino acid 281 of GAD_{67}, as well as the region between amino acid 78 and amino acid 97, or between amino acid 247 and amino acid 266, or between amino acid 335 and amino acid 356, or between amino acid 479 and amino acid 498, or between amino acid 509 and amino acid 528, or between amino acid 524 and amino acid 543, or between amino acid 539 and amino acid 556, or between amino acid 564 and amino acid 583 of GAD_{65}, as long as a substantial part of a given polypeptide is characterized by an amino acid sequence from that region, or segments or combinations thereof, and the polypeptide demonstrates the desired immunological or biological activity against autoimmune disease. In addition, polypeptides according to this invention include those having amino acid sequences which are longer or shorter in length than those of the polypeptides illustrated in Table 2 and Table 9, or which comprise segments or combinations thereof, as long as such polypeptides consist substantially of the region between the amino acids illustrated in Table 2 and Table 9 and demonstrate immunological or biological activity. All polypeptides of the invention should not stimulate or enhance the autoimmune disease.

Accordingly, it should be understood that the specific selection of any one polypeptide within the polypeptides of the invention does not involve undue experimentation. Such a selection may be carried out by taking a number of polypeptides and testing them for their immunological and biological activity in ameli rating the autoimmune disease or for detecting

antibody. The NOD mouse represents an excellent and well characterized model for screening polypeptides of the invention capable of ameliorating or preventing diabetes. Example 7 illustrates an acceptable procedure for routine screening of candidate polypeptides with biologic activity.

The polypeptides according to the present invention may be prepared by recombinant techniques or by conventional synthesis using known polypeptide synthetic methods, including synthesis on a solid support. An example of a suitable solid phase synthetic technique is that described by Merriweather (*J.Am.Chem.Soc.*, 85:2149, 1963). Other polypeptide synthetic techniques may be found, for example, in Bodanszky, *et al.*, *Peptide Synthesis*, John Wiley & Sons, 2d ed., 1976, as well as other references known to those skilled in the art. A summary of polypeptide synthesis techniques can be found in Stewart, *et al.*, *Solid Phase Peptide Synthesis*, Pierce Chemical Company, Inc., Rockford, Ill., 1984. The synthesis of polypeptides by solution methods may also be used, for example, as described in *The Proteins*, Vol. II, 3d ed., Neurath, *et al.*, eds., 105, Academic Press, New York, NY, 1976. Appropriate protective groups for use in such synthesis can be found in the above references as well as in J. McOmie, *Protective Groups in Organic Chemistry*, Plenum Press, New York, NY, 1973.

The polypeptides of the invention may also be prepared in an appropriate host transformed with DNA sequences that code for the desired polypeptide. For example, a polypeptide may be prepared by the fermentation of appropriate hosts that have been transformed with and which express a DNA sequence encoding th polyp ptide. Alternatively, a DNA sequence encoding several of the polypeptides of this inv ntion may be linked tog ther and thos sequences may then be used to transform an appropriate host to

permit the expression of polypeptides involved in the autoimmune disease.

The dosage ranges for the administration of the GAD polypeptides of the invention are those large enough to produce the desired effect in which the symptoms or cellular destruction of the autoimmune response are ameliorated. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex, and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary from about 0.1mg/m^2 to about 2000mg/m^2, preferably about 0.1mg/m^2 to about 500mg/m^2/dose, in one or more dose administrations daily, for one or several days.

The GAD polypeptides of the invention can be administered parenterally by injection or by gradual perfusion over time. The GAD polypeptides of the invention can be administered intravenously, intraperitoneally, intra-muscularly, subcutaneously, intracavity, transdermally, intranasally, or enterally.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenish rs (such as those based on Ringer's dex-

trose), and the like. Preservatives and other additives may also be pres nt such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

The invention also relates to a method for preparing a medicament or pharmaceutical composition comprising the GAD_{65} polypeptides of the invention, the medicament being used for therapy of autoimmune response to GAD_{65}.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided herein for purposes of illustration only and are not intended to limit the scope of the invention.

EXAMPLE 1

CLONING AND EXPRESSION OF GAD_{65}

A. RECOMBINANT DNA PROCEDURES

In order to obtain cDNA probes specific for GAD_{65} and GAD_{67}, total RNA was extracted from adult rat brain by guanidine isothiocyanate-cesium gradient using the method of Chirgwin, *et al.* (*Biochemistry*, 18:5294, 1979). Poly (A) RNA was purified on oligo dT cellulose, using the protocol by Bethesda Research Laboratories (BRL). First strand synthesis was performed by using MMLV-reverse transcriptase (BRL), with conditions suggested, except that poly $d(N_6)$-mers (Pharmacia) were used as primers. This cDNA-RNA mixture was heat inactivated at 65°C for 15 min and stored at -20°C. For PCR, 1/50 of the sample was added to the 100μl reaction. Degenerate oligonucleotides were synthesized (Applied Biosystems) to encode the underlined common amino acid sequ nces of feline (from cDNA) (Kobayashi, *et al.*, *J.Neurosci.*, 7:2768, 1987) and rat (from peptides) (Chang and Gottlieb,

J.Neurosci., 8:2123, 1988) GAD (Figure 1). The 5'-end sequence of each degenerate oligonucleotide contained one strand of the DNA sequence recognized by either SstI and HindIII (5' oligo) or SstI and SstII (3'-end oligo). These primers were used for selective amplification by polymerase chain reaction of the generated cDNA template as described by Gould, *et al.* (*Proc.Natl.Acad.Sci.,USA*, 86:1934, 1989). PCR products were subcloned into HindIII/SstI double digested Bluescript SK vector (Stratagene), transformed into DH5 (BRL), and plated by standard methods (Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989).

Colony hybridization was done with an 5'-^{32}P end labeled oligonucleotide specific to feline GAD_{67} (Kobayashi, *et al.*, *J.Neurosci.*, 7:2768, 1987). End labeling of oligonucleotide, hybridization conditions, and washing conditions were done as described (Wallace, *et al.*, in *Guide to Molecular Cloning Techniques*; Berger, *et al.*, Eds. in *Methods of Enzymology*; Abelson, *et al.*, Eds. *Academic Press, Inc.*, San Diego, 432-442, 1987), except that the nitrocellulose filters were washed at 50°C for 15 min. Colonies which were positive and negative in the hybridization were individually picked and grown overnight in Terrific Broth (Tartof, *et al.*, *Focus*, 9:12, 1987). DNA was isolated using a boiling method (Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989) and templates were denatured by 0.2N NaOH and purified by Sephacryl S400 spun columns (Pharmacia). Sequencing of denatured double stranded template was by the chain-t rmination method (Sanger, *et al.*, *Proc.Natl.Acad.Sci.,USA*, 74:5463, 1977) using the T7-sequencing kit (Pharmacia).

As shown in Figure 1, PCR-generated rat GAD_{65} and GAD_{67} cDNAs were used as probes to screen a lambda ZAP (Stratagene) rat hippocampus library provided by S. Heinemann (Salk Institute) by standard techniques (Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). A 2400 nucleotide GAD_{65} cDNA (the largest clone) was isolated and subcloned by "zapping" as described by Stratagene. When a rat GAD_{67} cDNA was obtained which was smaller than a 3.2kb rat GAD_{67} cDNA clone already on hand, the larger cDNA was sequenced. Exo III deletions (Henikoff, *Gene*, 28:351, 1984) were made in both directions for GAD_{65} and GAD_{67} and templates were prepared and sequenced as described above. Anchored PCR (Frohman, *et al.*, *Proc.Natl.Acad.Sci.,USA*, 85:8998, 1988) was done to clone the remaining 5'-ends of GAD_{65} and GAD_{67} mRNAs which were not represented in the original cDNA clones isolated in the library screening. Sequencing of these clones revealed that neither GAD_{65} nor GAD_{67} mRNAs contained any further initiation codons (AUGs) in frame with the previously designated initiation codons of the original cDNA clones.

EXAMPLE 2

CHARACTERIZATION OF CLONED GAD_{65}

A. NORTHERN BLOT HYBRIDIZATION

Two PCR-derived cDNA probes were hybridized to Northern blots containing rat brain RNA in order to determine whether the GAD_{67} and GAD_{65} cDNAs were derived from two different mRNAs. RNA was extracted as described in Example 1. Poly (A) RNA was separated by electrophoresis in formaldehyde and transferred onto Biotrans (ICN) membranes, and hybridization was performed as described by Well, *et al.* (*J.Neurosci.*, 16:311, 1986), except that 100µl/ml of poly (A) was

add d. Probes were labeled to approximately 10^9 dpm/μg by the oligolabeling procedure of Feinberg and Vogelstein (*Anal.Biochem.*, 132:6, 1983). Identical results were subsequently obtained with full-length clones of GAD_{65} and GAD_{67} cDNAs.

As shown in Figure 5, lanes 1 and 2 contain 1μg of poly (A) selected RNA extracted from rat cerebellum. Lane 1 was hybridized to a cDNA probe for the rat cognate of feline GAD_{67} (Kobayashi, *et al.*, *J.Neurosci.*, 7:2768, 1987) and lane 2 with a cDNA probe for the rat peptide sequence (which corresponds to GAD_{65}).

The cDNA probe for the rat peptide sequence hybridized to a 5.7kb RNA, while the cDNA probe for the rat cognate of feline GAD_{67} cDNA, hybridized to a 3.7kb RNA. This demonstrates that GAD_{65} and GAD_{67} are not derived from the same mRNA.

B. **GENOMIC HYBRIDIZATION OF GAD_{67} AND GAD_{65}**

In order to investigate the possibility that GAD_{67} and GAD_{65} arise from separate genes, cDNAs of both GAD_{67} and GAD_{65} were hybridized to DNA blots containing genomic DNA.

For Southern blots, genomic DNA was extracted from rat liver as described (Kaiser, *et al.*, in *DNA Cloning*, vol.I, A Practical Approach, D.M. Glover ed., IRL Press, Oxford, pp. 38-40, 1985). DNA (10μg/sample) was digested to completion with EcoRI and HindIII using conditions recommended by the suppliers (BRL, Gaithersburg, MD). DNA fragments were separated by electrophoresis at 1.5v/cm for 16 hrs in 0.8% agarose. The DNA was then transferred to Zeta-Probe membranes (Bio-Rad), hybridized, and washed, as described by Gatti, *et al.* (*Biotechniques*, 2:148, 1984), except that 5μg/ml Carnation dried milk was substituted for Denhardt's solution. Probes for Southern blots were labeled as d scribed in Example 1, above.

As shown in Figure 6, genomic DNA digested with HindIII and EcoRI are in lanes 1 and 3 and lanes 2 and 4, respectively. GAD_{67} cDNA was hybridized to lanes 1 and 2, whereas GAD_{65} cDNA was hybridized to lanes 3 and 4. Numbers along the side of the gel are the DNA fragment sizes in kilobases.

This data shows that the two cDNAs hybridize to genomic fragments of different sizes. In addition, the greatest contiguous stretch of identical nucleotide sequence of GAD_{65} and GAD_{67} cDNAs is only 17 nucleotide bases in length. Thus, GAD_{67} and GAD_{65} are encoded by two distinct genes.

C. ENZYMATIC COMPARISON OF GAD_{67} AND GAD_{65}

Studies were done comparing the effect of PLP on the activity of GAD_{67} and GAD_{65}. In so doing, both cDNAs were subcloned into vectors that allowed their expression in bacteria (Studier, *et al.*, *J.Mol.Biol.*, 189:113, 1986). Overexpression of "fusionless" GAD_{65} and GAD_{67} was accomplished by subcloning GAD_{65} cDNA into the NcoI site of pET-8c and GAD_{67} cDNA into the NheI site of pET-5c vectors (Studier, *et al.*, *J.Mol.Biol.*, 189:113, 1986).

To obtain compatible sticky ends for correct in-frame subcloning of both cDNAs, selective amplification was performed by PCR using conditions suggested by United States Biochemical (USB), with 200μM dNTPs and 1.5mM $MgCl_2$ in the mixture and annealing at 55°C with 20 cycles to decrease infidelity of AmpliTAQ (USB). Primers specific for GAD_{65} and GAD_{67} contained one DNA strand of the NcoI and SpeI recognition sites, respectively. Since there is a NheI restriction site within the coding region of GAD_{67}, SpeI (which is compatible with NheI) was used.

PCR products were subcloned into their respective pET vectors, transformed into DH5 and plated as described (Maniatis, *et al.*, *Molecular Cloning: A*

Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). Colonies were picked and grown overnight in LB broth with 50μg/ml ampicillin. Subclones with correct orientation were transformed into BL21(DE3) strain (Studier, *et al.*, *J.Mol.Biol.*, 189:113, 1986) for overexpression. As a negative control, the pET-8C vector with no insert was transformed and subsequently induced. Single colonies were picked, grown, induced by 1mM isopropyl-B-D-thiogalacto-pyranoside (IPTG), and analyzed on SDS-PAGE gels as described (Sambrook, *et al.*, *Molecular Cloning a Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 17.15-17.16, 1989).

To measure GAD activity, we induced 10ml cultures of bacteria at OD_{600}-0.5 with 1mM IPTG. Two hours after induction, bacteria was spun down and resuspended and sonicated in 1ml of homogenizing buffer (1mM phenylmethylsulfonyl fluoride (PMSF), 1mM 2-aminoethylisothiouronium bromide (AET), and 60mM potassium phosphate, pH 7.1). After sonication, cell debris was removed by centrifugation and protein concentration was measured (Bradford, *Anal.Biochem.*, 72:248, 1986) in the supernatant (supernatant was stored in aliquots at -70°C). Brain homogenates were prepared as described (Legay, *et al.*, *J.Neurochem.*, 46:1478, 1986). GAD activity was measured as described (Krieger, *et al.*, *J.Neurochem.*, 33:299, 1984) with 0.2mM PLP present or absent and 20μl of brain homogenate or bacterial lysate in the incubation mixture. Production of $^{14}CO_2$ in bacterial lysates was linear with respect to time of incubation and protein concentration.

TABLE 3

Source	GAD Specific Activity* - PLP	GAD Specific Activity* + PLP	Fold Increase in Induction
BL21(DE3) + pET-8c	12 ± 0.4	9 ± 1	--
BL21(DE3) + pET-GAD_{65}	115 ± 3	773 ± 61	6.7
BL21(DE3) + pET-GAD_{67}	160 ± 2	389 ± 8	2.4
Rat Brain	131 ± 5	216 ± 2	1.6

* cpms of $^{14}CO_2$/µg protein/hr of triplicates ± S.E.M.

As shown in Table 3, bacterial lysates containing GAD_{65} or GAD_{67} catalyze the conversion of [1-^{14}C]-glutamate to GABA and $^{14}CO_2$.

PLP stimulates the enzymatic activity of GAD_{65} more than GAD_{67}. This greater stimulation probably reflects the faster cycling of GAD_{65} through the inactivation cycle proposed by Martin and coworkers (Martin, *Cell.Mol.Neurobiol.*, 7:237, 1987). This faster cycling suggests that GAD_{65} contributes more to the pool of apo-GAD that exists *in vivo* (Miller, *et al.*, *Brain Res.Bull.*, 5(Suppl.2):89, 1980). Thus, *in vivo*, PLP appears to regulate GAD_{65} activity more than GAD_{67} activity.

GAD_{65} activity in bacterial lysates is similar to the five-fold PLP stimulation of GAD activity found in synaptosomes prepared from rat substantia nigra (Miller, *et al.*, *J.Neurochem.*, 33:533, 1979). Because both GADs are more dependent upon added PLP in bacteria than is the GAD activity in crude rat brain homogenates, the endogenous PLP concentration of bacteria lysates may be less than rat brain homogenates.

D. IMMUNOL GICAL IDENTIFICATION OF GAD_{65} AND GAD_{67}

Rat brain homogenates and bacterial lysates were extracted as described above. Equal volumes of loading buffer were added to each sample as described (Harlow, *et al., Antibodies, A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988). Proteins were separated by electrophoresis in a 10% acrylamide gel in SDS and electrophoretically transferred to nitrocellulose (Harlow, *et al., Antibodies, A Laboratory Manual,* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988). The unreacted sites were blocked with a phosphate buffered saline (PBS) solution containing 2% bovine serum albumin (fraction V), 1% gelatin, and 1% Triton-X-100 at 42°C for one hr. After washing, the nitrocellulose filter was then cut into three sections and incubated with the following primary antibodies: lanes 1 to 4 with a 1/2000 dilution of the antiserum of Oertel, *et al.* (*Neuroscience*, 6:2689, 1981), which recognizes both GAD_{67} and GAD_{65}; lanes 5-8 with a 1/2000 dilution of K-2 antiserum, which recognizes only GAD_{67}; lanes 9-12 with a 1/2000 dilution of GAD-6 monoclonal antibody, which is specific for GAD_{65} (Chang, *et al., J.Neurosci.*, 8:2123, 1988). All filters were extensively washed and appropriate secondary antibodies were incubated and washed. Bound antibodies were detected with ^{125}I-labeled protein A and autoradiography. Each lane contained the following: lanes 1, 5, and 9 are BL21(DE3) + pET-GAD_{67}; lanes 2, 6, and 10 are BL21(DE3) + pET-GAD_{65}; lanes 3, 7, and 11 are rat brain homogenate; and lanes 4, 8, and 12 are BL21(DE3) + pET-8c.

The immunoblots of bacterially produced GAD_{65} and GAD_{67} demonstrated that GAD_{65} indeed corresponds to the smaller GAD in brain extracts, and GAD_{67} to the larger form (Figure 7). Previous work has demonstrated the

correspondence of GAD_{67} to the larger GAD for feline GAD_{67}, and for mouse GAD_{67} (Katarova, *et al.*, *Eur.J.Neurosci.*, 2:190, 1990; 235, 1987). The mobilities of bacterially produced GAD_{65} and GAD_{67} (as detected with the antiserum of Oertel, *et al.* (*Neuroscience*, 6:2689, 1981) are identical to the immunoreactive doublet seen in rat brain homogenate.

The smaller molecular weight and larger molecular weight forms of GAD in rat brain are thus identical in antigenicity and size to the products of GAD_{65} and GAD_{67} cDNAs, respectively. Consequently, the two GADs in rat brain are GAD_{65} and GAD_{67}. From these data it can also be concluded that the molecular identity of the reported PLP-dependent and PLP-independent GADs by Tapia (Bayon, *et al.*, *J.Neurochem.*, 29:519, 1977) are GAD_{65} and GAD_{67}, respectively. Martin and coworkers (Spink, *et al.*, *Brain Res.*, 421:235, 1987) have reported the existence of four kinetically different forms of rat brain GAD. However, immunoblotting experiments (with the antisera used here) of these forms have not been reported.

E. DISTRIBUTION OF GAD_{65} and GAD_{67} IN RNAs IN BRAIN TISSUE

Experiments were done to determine the distribution of GAD_{65} and GAD_{67} in RNAs in cerebellum using *in situ* hybridization.

Transcripts of, respectively, 3.2kb and 2.3kb from GAD_{65} and GAD_{67} cDNAs, were radiolabeled with ^{35}S according to Wuenschell, *et al.* (*Proc.Natl.Acad.Sci.,USA*, 83:6193, 1986) procedure. Hydrolyzed fragments of 200 bp were hybridized to coronal sections of a rat cerebellum. Animals were anesthetized under halothane and decapitated. The brain was rapidly frozen in dry ice and coronal frozen sections (12μm) were fixed for 30 min in freshly prepared 4% formaldehyde in phosphate-buffered saline

(PBS; 130mM NaCl, 10mM Na phosphate, pH 7.0). The tissue was dehydrated through graded ethanol solutions and stored at -70°C.

In order to increase tissue permeability, the sections were submitted to the following pretreatments: rehydration through graded ethanol solutions (5 min each in 95%, 85%, 70%, 50%, and 30% ethanol); PBS (5 min); 0.02N HCl (10 min); PBS (5 min); 0.01% Triton N-101 in PBS (1 min); PBS (2 x 5 min); 1μg/ml proteinase K (7.5 min); and glycine (to inhibit proteinase K) in PBS (3 x 5 min). Proteinase K was digested for 30 min at 37°C before use. Sections were then incubated at 37°C in 50% formamide, 750mM NaCl, 25mM EDTA, 0.2% SDS, 0.02% BSA, 0.002% Ficoll, 0.02% polyvinylpyrrolidone, 250μg/ml yeast tRNA, 250μg/ml poly A, and 25mM PPES (pH 6.8).

For the hybridization, 100mM DTT, 10% dextran sulfate, and sense or antisense ^{35}S-RNA were added to the prehybridization solution. An aliquot (50μl) of the hybridization solution containing about 3 ng (10^6 cpm) of probe (sense or antisense) was added onto the slides. Each slide was coverslipped and incubated for 16 hrs at 50°C, following which the siliconized coverslips were removed by brief washing in 4 x SSC (1 x SSC - 150mM NaCl, 60mM Na citrate, pH 7.0).

Sections were then treated with ribonuclease A (50μg/ml in 0.5M NaCl, 10mM Na thiosulfate, 1mM EDTA, 10mM TrisHCL, pH 8.0) for 20 min at 37°C and rinsed for 2 hrs at room temperature in 2 x SSC, 10mM Na thiosulfate, for 30 min at 55°C. Sections were dehydrated in ethanol, delipidated in xylene, coated with Kodak NTB2 emulsion and exposed for 10 days at 4°C. The emulsion was developed with Kodak D19, and the tissu counterstained with cresyl violet.

Autoradiographic grains were detected using reflected polarized light and grain numbers, densities, nd cell areas were determined with an Analytic Imaging

Concepts image analyzer system. Due to the low background level, the criteria for defining a cell "labeled" was based on the presence of more than 5 clustered grains. The GAD labeled cells were found scattered throughout the brain, enabling the measurement of the number of grains over individual cells. The boundary of the cell and the area covered by a grain allowed the calculation of the number of grains per cell. This analysis was done at a high magnification (800X), using both reflected polarized light and transmitted light to simultaneously visualize the stained cell and the superimposed grains. Numbers are means ± S.E.M. of "n" cells.

TABLE 4

	GRAINS/CELL		
CELL TYPE	GAD_{67} mRNA	GAD_{65} mRNA	GAD_{67}:GAD_{65}
Purkinje	172 ± 34 (87)*	43 ± 2 (70)	4.0
Golgi II	96 ± 8 (80)	64 ± 9 (65)	1.5
Basket	61 ± 12 (102)	16 ± 1 (57)	3.8
Stellate	55 ± 15 (65)	18 ± 3 (37)	3.1

* ± S.E.M.(n)

In all neuronal types GAD_{67} mRNA levels are greater. The observations with *in-situ* hybridization are consistent with previous findings (Nitsch, *J.Neurochem.*, 34:822, 1980; Denner, *et al.*, *J.Neurochem.*, 44:957, 1985; Itoh, *et al.*, *Neurochem. Res.* 6:1283, 1981) that the ratio of PLP dependent to independent GAD activities in the cerebellum is one of the lowest in brain regions tested. In addition, as shown in Table 3, the order of amounts for GAD_{67} mRNA is Purkinje > Golgi II > Basket > Stellate cells; in contrast, for GAD_{65} mRNA, this order is Golgi II > Purkinje > Bask t > Stellate cells.

The expression of GAD_{65} and GAD_{67} mRNAs thus differs among class s of neurons. The contribution of each to

total GAD activity in turn affects how GABA production is regulated. For example, the substantia nigra contains one of the highest ratios of PLP-dependent to PLP-independent GAD activities (Nitsch, *J. Neurochem.*, 34:822, 1980). Increasing GABA concentration in the substantia nigra by local injection of inhibitors of GABA catabolism is especially effective in reducing seizure susceptibility (Gale, *Fed. Proc.*, 44:2414, 1985). Experimental animals undergoing seizures induced by PLP-antagonists may therefore be unable to inhibit seizure propagation because of inhibition of GAD_{65} particularly in nerve terminals within the substantia nigra.

F. SUBCELLULAR LOCATION OF GAD_{65} AND GAD_{67}

The distribution of GAD_{65} and GAD_{67} was evaluated in the S_2 and synaptosome subcellular fractions. S_2 is a high speed supernatant consisting of the cytosol of all cells in the brain, while the synaptosomal fraction consists primarily of nerve endings (Gray, *et al.*, *J. Anat., Lond*, 96:79, 1962). For these studies, whole rat brain fractionation was performed as described by Booth and Clark (Booth, *et al.*, *Biochem. J.*, 176:365, 1978). Protein concentrations were determined by Schaffner and Weissman (Schaffner, *et al.*, *Anal. Biochem.* 56:502, 1973). Samples were prepared as described (Kaiser, *et al.*, *DNA Cloning, Vol. I, A Practical Approach, D.M. Glover ed.* (IRL Press, Oxford, 1985, pp. 38-40), and immunoblotting was done as described above using GAD-6 monoclonal antibody and K-2 antiserum. Equal amounts of protein (16μg) were added to each lane. Autoradiography showed a linear response of increasing amount of ^{125}I-protein A bound to antibody with protein concentrations of 1, 3, 10, 30, 100μgs with both K-2 antiserum and GAD-6 monoclonal antibody (data not shown).

The results showed that GAD_{67} was present in equal amounts in both fractions. Since the S_2 fraction contains the cytosolic proteins of glial (as well as other non-neuronal) and neuronal cells, the concentration of GAD_{67} must be greater in neuronal cell bodies than in nerve endings. In contrast, the concentration of GAD_{65} was greater in synaptosomes than in S_2. These subcellular fractionation experiments suggest that, in contrast to GAD_{65}, a much greater fraction of GAD_{67} is present in cell bodies of neurons than in nerve terminals. Thus, subcellular fractionation, like immunohistochemistry, shows that GAD_{65} and GAD_{67} have different subcellular distributions.

In vivo experiments utilizing inhibitors of GABA synthesis and degradation have suggested that the GABA pool in neuronal cell bodies is different from that in the nerve terminals (Iadarola, *et al.*, *Mol. Cell. Biochem.*, 39:305, 1981). GABA produced by GAD_{67} may be involved more in cellular metabolism (for example, in the GABA shunt) and in dendrodendritic synapses. The dendrites of granule cells in the olfactory bulb, which form dendrodendritic synapses with mitral dendrites (Shepard, *Physiol. Rev.*, 52:864, 1972) and probably release GABA (McLennan, *Brain Res.*, 29:177-184, 1971), label intensely with K-2 antiserum. While not shown here, it has also been found greater GAD_{67} than GAD_{65} mRNA levels (2-3 fold) in the olfactory bulb. This distribution is consistent with the reported finding that most GAD activity in the olfactory bulb is present in S_2 and P_1 (crude nuclear pellet) and not in synaptosomes (Quinn, *et al.*, *J. Neurochem.*, 35:583, 1980).

The differing subcellular distributions of GAD_{65} and GAD_{67} could result from cytoskeletal anchoring or from some unknown protein targeting mechanism. Some cyt skel tal proteins have distributions that resemble GAD_{65} and GAD_{67}. For example, in cultured sympathetic

neurons Peng, *et al.* (*J Cell. Biol.*, 102:252, 1986), demonstrate that 84% of tau is in axons while 100% of MAP-2 is in cell bodies and dendrites. In addition, 43kd protein, a cytoskeletal protein, is thought to anchor the acetylcholine receptor to the underlying membrane cytoskeleton (Flucher, *et al.*, *Neuron*, 3:163, 1989).

EXAMPLE 3

DETECTION OF GAD AUTOANTIBODIES IN CLINICAL SPECIMENS

A. MATERIALS AND METHODS

1. Patient Specimens. Sera from four groups of individuals were selected from a previous study by Atkinson and co-workers (Atkinson, *et al.*, *Lancet*, 335:1357-1360, 1990). These groups consisted of: Group (1), 1 new onset IDD patients diagnosed according to the established National Diabetes Data Group (NDDG) criteria (Gleichman, *et al.*, *Diabetes*, 36:578-584, 1987) that had been referred to the University of Florida, Diabetes Clinics; Group (2), 5 randomly selected islet cell cytoplasmic antibody (ICA) negative non-diabetic controls without any known family history of autoimmune disease; Group (3), 13 individuals whose sera had been collected 3 to 66 months prior to their documented clinical onsets of IDD; Group (4), non-diabetic controls and relatives, and those who were studied prior to their onsets of IDD; and Group (5), 3 patients at risk for IDDM, but where onset has not yet occurred. This latter group had been ascertained through ongoing prospective ICA screening studies of more than 5000 first degree relative of IDD probands, and 8200 individuals from the general population (of which 4813 were school children).

2. **Islet Cell Autoantibodi s**. ICA were assayed by indirect immunofluorescence on blood group O cryocut pancreatic (Atkinson, *et al.*, *Lancet*, 335:1357-1360, 1990). All results were interpreted on coded samples, with control negative and positive sera in each batch. The degrees of ICA positivity were analyzed with the guidelines established by the Immunology Diabetes Workshop (IDW) for the standardization of ICA (Gleichman, *et al.*, *Diabetes*, 36:578-584, 1987). All positive sera were titered by end point dilution, and the Juvenile Diabetes Foundation (JDF) units were determined by reference to a standard serum previously calibrated to the international JDF standard of 80 units. In the studies reported here, a positive ICA result was defined by replicate titers of 10 JDF units or greater.

3. **HLA DR Typing**. HLA DR typing was performed as adapted from the method described by Van Rood and Van Leuwen (*Nature*, 262:795-797, 1976), using DR trays (One Lamda Laboratories, Los Angeles, CA).

4. **Human Islet Cells**. Human pancreatic islets were isolated from cadaveric pancreases and maintained *in vitro* as previously described (Ricordi, *et al.*, *Diabetes*, 37:413-420, 1988). The islet cells were metabolically labeled with ^{35}S methionine (Amersham, Arlington Heights, IL) *in vitro* (95% air/5%CO_2).

5. **Islet Cell Extractions and Immunoprecipitations**. Islet cells were extracted as previously described by Atkinson, *et al.* (*Lancet*, 335:1357-1360, 1990) with the following modifications. For immunoprecipitation studies, the islet cell lysates were precleared twice by incubation (2h, 4°C) with either control, IDD serum (100µl), or GAD-6 (Chang, *et al.*, *J.Neuro*, 8:2123-2130, 1988) (1µl in 99µl of Tris buffer (Atkinson, *et al.*, *Lanc t*, 335:1357-1360, 1990) for very 1000 islets. Immun complex s were then absorbed (1h 4°C) with an

excess of protein A Sepharose CL-4B (Pharmacia, NJ). Aliquot volumes representing 1000 islet cells containing unbound (precleared) lysate were then incubated (12h, 4°C) with either IDD or control sera (25μl), or GAD-6 (Chang, *et al.*, *J.Neuro*, 8:2123-2130, 1988) (1μl in 25μl Tris buffer). Following another incubation with protein A Sepharose CL-4B (1h, 4°C), the complexes were then washed 5 times with 50mM Tris HCL (pH 7.4) with 0.1% SDS, 1.0% Triton X-114, and 2mM EDTA, and then washed again one time in double distilled water. The protein A Sepharose CL-4B was then boiled in Laemmli sample buffer (Laemmli, *Nature*, 227:680-685, 1970), and the samples were subjected to SDS-PAGE and fluororadiography (Kodak, X-omat AR5) using Enhance (New England Nuclear). Alternatively, the autoradiographs were analyzed by a BETAGEN (Boston, MA) analyzer. Both 64KA positive and negative sera were used in each assay, to serve as interassay controls. All fluororadiographs were analyzed and rated as positive or negative after comparison with the known interassay controls. Positive serum samples were designated as 1 when a sample resulted in immunoprecipitation of a low intensity 64,000 M_r band, 2 if a moderate intensity band was observed and 3 if the intensity of the immunoprecipitated protein was high. A similar rating procedure was employed for the intensity of bands corresponding to immunoprecipitated ^{35}S-GAD_{65} and ^{35}S-GAD_{67}.

6. **Immunoprecipitations**. Immunoprecipitation of bacterial lysates containing ^{35}S-GAD_{65} or ^{35}S-GAD_{67}, and GAD from human brain homogenate, was completed as described above in immunoprecipitation studies of human islet cell extractions.

7. **GAD Assays**. Human brain homogenat s were incubated with patient sera as described above in human islet cells. Aft r absorption and washes, the protein A agarose slurry was aliquoted into three equal volumes

and GAD activity was measured as described (Krieger, *et al., Neurochem.* 33:299, 1984). Briefly, Protein A agarose beads were incubated with (1-^{14}C)-glutamate (Amersham) in a designated incubation mixture (Krieger, *et al., J. Neurochem.* 33:299, 1984) and production of $^{14}CO_2$ was quantitated by a liquid scintillation counter.

8. **Production of ^{35}S-GAD$_{65}$ and ^{35}S-GAD$_{67}$.** Rat GAD$_{65}$ and GAD$_{67}$ cDNAs were subcloned into a bacterial expression system as previously described. Labeling of ^{35}S-GADs was completed by pulsing IPTG induced bacterium (growing in Minimal Media) for 15 minutes with TRAN ^{35}S-label (ICN). Cultures were then spun down and resuspended and sonicated in 1ml of homogenizing buffer (1mM phenylmethylsulfonyl fluoride (PMSF), 1mM 2-aminoethylisothiouronium Bromide (AET) and 60mM potassium phosphate, pH 7.1). After sonication, cell debris was removed by centrifugation and protein concentration was measured (Bradford, *Anal.Biochem.*, 72:248, 1986) in the supernatant (supernatant was stored in aliquots at -70°C).

B. IMMUNOREACTIVITY OF IDDM SPECIMENS

Sera from patients with IDDM were tested for the ability to precipitate GAD from human brain homogenates.

TABLE 5

SERA FROM IDDM PATIENTS IMMUNOPRECIPITATE GAD ACTIVITY

Patient	IDDM	Pre-IDDM Period[1]	64K[2]	JDF[3]	GAD Activity[4] cpm's
DA	*[5]	>24	3	164	13,762
DC	*	>1	3	20	1,719
RS	+	5	3	40	588
NL	+	0	2	80	440
DM	*	>1	2	10	184
C	-	na	0	0	280
C	-	na	0	0	285
C	-	na	0	0	325
C	-	na	0	0	275
C	-	na	0	0	270

[1] Expressed as months
[2] 64K titers as described in Experimental Methods
[3] The islet cell antibody test as expressed in Juvenile Diabetes Foundation (JDF) units
[4] Not adjusted for background
[5] At risk for diabetes (also, failed glucose test)
na - Not applicable

As shown in Table 5, the sera of four (out of five) at risk for IDDM or IDDM patients bound significantly greater amounts of enzymatically active GAD of human brain extracts than sera from control patients. In addition, sera from one of the patients was drawn in a pre-IDDM period, thus autoantibodies to GAD are present prior to the onset of IDDM symptoms (see C below).

Further experiments (results not presented) showed that the sera of two at risk IDDM patients (DA, DC) immunoprecipitated recombinantly produced ^{35}S-GAD_{65} whereas recombinantly produced ^{35}S-GAD_{67} was only recognized by sera of patient DA (and to a lesser degree than ^{35}S-GAD_{65}).

Additional studies using patient DA sera showed the presence of antibodies which recognize specific polypeptides produced in human pancreatic islet cells. Electrophoretic analysis of the bound polypeptides demonstrated the presence of autoantibodies to a 64kD component, as previously shown by others in human IDDM (Baekkeskov, *et al.*, *Nature*, 298:167-169, 1982) and in animal models (Baekkeskov, *et al.*, *Science*, 224:1348-1350, 1984; Atkinson, *et al.*, *Diabetes*, 37:1587-1590, 1988). Prior absorption of these sera with GAD-6 monoclonal, which recognized GAD_{65} but not GAD_{67}, or with bacterially produced GAD_{65}, abolished the ability of the sera to recognize the 64kD pancreatic polypeptide. The epitopes recognized by autoantibodies to the 64kD autoantigen are thus present in GAD_{65}, indicating that the 64kD autoantigen is indeed GAD_{65}. In order to investigate the predictive value of GAD_{65}, sera drawn from patients prior to onset of clinical manifestation of IDDM were tested for autoantibodies to GAD_{65}.

TABLE 6

IDDM PATIENTS ANALYZED FOR AUTOANTIBODIES PRIOR TO THE ONSET OF DISEASE

Patient	Sex	HLA	Age Onset[1]	Pre-IDD Period[2]	JDF	64KA[3]	GAD^{3}_{65}	GAD^{3}_{67}
TA	M	3,2	17	11	20	2	0	1
CA	F	4,5	38	4	0	1	1	0
RA	M	2,1	5	34	0	2	1	0
TB	M	2,4	11	66	40	1	1	0
AB	M	N.D.	23	6	160	3	3	2
VC	F	4,6	15	3	40	1	0	1
JD	M	6,1	34	25	10	3	1	1
DR	F	3,4	14	42	320	2	1	0
JG	M	3,3	12	8	40	1	0	0
BR	M	3,3	5	9	0	0	1	1
KR	F	4,X	34	14	10	3	2	0
JT	F	4,6	7	10	N.D.	1	1	1

[1] Age of IDDM onset expressed as months
[2] The time interval between sera drawn and IDDM onset expressed as months
[3] 1 = lowest; 2 = medium; and 3 = highest band intensities
N.D. - not determined

As shown in Table 6, 9 out of 12 specimens (75%) were immunoreactive with ^{35}S-GAD_{65}. In addition, two patients (JA and VC) were immunoreactive to GAD_{67}, but not GAD_{65} under these conditions. Therefore, in combination, autoantibodies to GAD_{65} and GAD_{67} were present in 11 out of 12 (91%) of these patients sera. This finding suggests that although autoantibodies to GAD_{65} are more common than autoantibodies to GAD_{67}, the use of both recombinant GADs (GAD_{65} and GAD_{67}) in an assay would allow for greater predictability of IDDM. Previous tests of these sera (Atkinson, *et al.*, *Langet*, 335:1357-1360, 1990) demonstrated that 11 out of 12, or 92%, immunoreacted with the ^{35}S-64kD molecule from human pancreatic islet cells. The serum which contained detectable autoantibodies to the 64kD molecule and not GAD_{65} was a serum which contained the lowest titer (or "1") for the 64kD molecule. Thus, the false negative obtained was due to a lack of sensitivity in this assay. Furthermore, this assay predicted IDDM in one patient (BR) who was negative for 64K.

These results show that the 64kD molecule identified in β-cells of human pancreas is identical in size and antigenicity to rat GAD_{65}. Furthermore, sera drawn from patients prior to IDDM onset contain autoantibodies to GAD_{65}. Consequently, the GAD_{65} recombinant molecule is of great utility as a diagnostic tool for predicting IDDM. The ability of a physician to diagnose IDDM prior to actual symptoms may result in a greater extension of time before insulin therapy is needed. The sensitivity of such immunoassays will improve with the use of a recombinant GAD_{65} of human origin which represents the GAD form present in β-cells of the pancreas.

EXAMPLE 4

IMMUNE PROLIFERATIVE RESPONSE TO POLYPEPTIDE

Polypeptides were synthesized using an automatic instrument (Applied Biosystems) and standard conditions. These polypeptides were then tested to compare their relative ability to stimulate proliferation of splenic lymphocytes and islet infiltrating T lymphocytes (IITLs). In this study, polypeptides derived from the GAD_{65} core sequence and from the homologous region of polio virus were compared. Appropriate cells were cultured for 5 days with the respective polypeptide in the presence of 5 X 10^4 irradiated spleen cells. ^{3}H-thymidine was added during the last 16 hours of culture.

TABLE 7

ANTIGEN	AMINO ACID SEQUENCE	^{3}H-THYMIDINE INCORPORATION (cpm) BY LYMPHOID CELL POPULATION	
		IITLs[a]	SPLEEN[b]
None	---	1,100	6,500
Poliovirus	MKSMCPQAQLKVKYL	900	22,500
GAD_{65}	ARFKMFPEVKEKGMAA	9,500	23,300

[a] islet infiltrating T lymphocytes (3 X 10^4 cells/well)

[b] 1 X 10^5 cells/well

In these studies, there was no significant difference in the proliferative activity of cultures of spleen lymphocytes exposed to either the polio or the GAD_{65} polypeptides. However, both polypeptides stimulated a T cell response which was higher than that found in the media control. The lack of difference in proliferation in the spleen cell population may be due to a lower frequency of GAD polypeptide specific T cells.

The IITL population, when evaluated in the same manner, showed a marked difference in cell proliferation. In this system, the response to the GAD_{65} polypeptide was 9-fold greater than that of either the culture media or the polio polypeptide. This data strongly suggests that the GAD_{65} is an important antigen for T cell responses in the IITL population. This data suggests that molecular mimicry plays a role in the pathogenesis of diabetes.

EXAMPLE 5

GAD INDUCES PROLIFERATION OF SPLEEN CELLS OF NOD MICE

Proliferative T-cell responses to β-cell antigens (βCA) develop spontaneously in the nonobese diabetic (NOD) mouse model in a defined chronological order. The NOD mouse experimental model is considered the most analogous *in vivo* system available for studying IDDM in humans. This example describes studies on the antigen-induced blastogenesis of spleen cells from newborn to 5 month old female NOD mice when exposed to GAD and other peptides.

The βCAs tested included one of the two forms of GAD (Kaufman, *et al.*, *Science*, 232:1138-1140, 1986; Erlander, *et al.*, *Neuron*, 7:91-100, 1991; Kaufman, *et al.*, *Trends in Pharm. Sci.* (in press)), (GAD_{65}, previously known as the 64K autoantigen (Baekkeskov, *et al.*, *Nature*, 298: 167-169, 1981; Baekkeskov, *et al.*, *Nature*, 347:151-156, 1990), carboxypeptidase H (CPH)

(Castano, *et al., J. Clin. Endoctrinol. Metab.*, 73:1197-1201, 1991), insulin (Palmer, Predicting IDDM, *Diabetes Reviews*, 1:104-115, 1993) and a peptide of hsp which has been shown to be the immunodominant determinant recognized by NOD T-cells (Elias, *Proc. Natl. Acad. Sci.*, 88:3088-3091, 1991). GAD in particular, is a good candidate for the initial target antigen in IDDM since autoantibodies to GAD arise early in the natural history of the disease (Baekkeskov, *supra*; Atkinson, *et al., Lancet*, 335:1357-1360, 1990; Kaufman, *et al., J. Clin. Invest.*, 89:283-292,1992). Furthermore, unlike the ubiquitous hsp, GAD is expressed primarily in β-cells and the immunologically privileged central nervous system (CNS) and gonads. As control antigens, irrelevant prototype foreign and self antigens including hen eggwhite lysozyme (HEL), human serum albumin (HSA), *E. coli*. β-galactosidase (β-gal) and murine myelin basic protein (MBP) were used.

NOD (Taconic farms) and BALB/c mice (Jackson Laboratories) were kept under specific pathogen free conditions. The mice were sacrificed at the ages indicated and the spleen cells were tested directly *ex vivo* for their proliferative recall response to antigen. Single cell suspensions of spleen cells were plated at 1 x 10^6 cells per well in 96 well microtiter plates in 200 μl serum free HL-1 medium (Ventrex) that was supplemented with 2mM glutamine with or without 10 μg/ml antigen (or 7 μM peptide) in triplicate cultures. During the last 16h of the 72h culture period, 1 μCi[^{3}H]-thymidine was added per well. Incorporation of label was measured by liquid scintillation counting.

Both human GAD_{65} (Bu, *et al., Proc. Natl. Acad. Sci.*, 89:2115-2119, 1992) and *E. coli* β-gal (control) were purified from recombinant bacteria on the basis of a hexahistidine tag which allows their rapid affinity purification by metal affinity chromatography (Hochuli, *et al., Bio/T chnology*, 6:1321-1325, 1988). Bovine CPH

was the generous gift of L. Fricker (Albert Einstein Col. Med.) and human insulin was purchased from Eli Lilly.

As illustrated in Figure 8, while proliferative T-cell responses were not detected at any time point to the control antigens, a response to GAD arose at 4 weeks of age in NOD mice, concurrent with the onset of insulitis in the colony. The blastogenesis induced by GAD increased during the next four weeks and then declined to background levels by week 16. At 6 weeks of age, near the peak of anti-GAD reactivity, T-cell responses to hsp appeared and increased until week 15 and then diminished as well (Figure 8). In all NOD mice tested, hsp reactivity was preceded by an anti-GAD response, suggesting that the former reactivity developed as a secondary event during the autoimmune process. Similarly, while no response was detected to CPH at 4 weeks of age, a strong anti-CPH response was observed by week 8. In some mice, a weak response to insulin was observed at 12 weeks, which became more prevalent at 15 weeks of age (Figure 8 and Table 8). None of the antigens induced proliferation in T-cells from age-matched control BALB/c or (NOD x BALB/c) F_1 mice, both of which do not develop insulitis or IDDM. T-cell reactivity subsequently arises to other βCAs, consistent with the inter-molecular diversification of the autoimmune response. Thus, the autoimmune response to GAD was the first to occur among the autoantigens tested. In view of this, tolerization to GAD should prevent the spread of autoimmunity to other βCAs and insulitis. If this were not the case then tolerization to GAD should have no effect on the response to these other antigens.

Blastogenesis provides an approximation of the relative clonal sizes of antigen-specific CD4+ T-cells (Corradin, *et al.*, *J. Immunol.*, 119:1048-1053, 1977). The data in Figure 8 shows that GAD reactive T-cells

"spontaneously" undergo clonal expansion concurrent with the onset of insulitis. These findings ar consistent with an endogenous priming event.

EXAMPLE 6

INDUCTION OF TOLERANCE WITH GAD

This example describes a study which shows that induced tolerance to GAD can ameliorate IDDM.

1. In these experiments female NOD mice were intravenously injected at 3 weeks of age with 50 μg GAD, β-galactosidase, mycobacterial hsp65 (m-hsp) or 0.1 μg of the immunodominant hsp peptide (hsp-p), in PBS. At 12 weeks of age, mice were examined for insulitis and autoantigen reactive T-cells. At this age both indications are established in untreated NOD mice. Pancreatic tissue sections were stained by immunoperoxidase techniques for insulin and were counterstained with hematoxylin. Insulitis was scored in a blinded manner by examining 54 to 87 islets on 5 interrupted tissue sections from each pancreas. Proliferative splenic T-cell responses induced by various antigens were performed as described above in Example 4. Data in Table 8 are expressed as the average [^{3}H]-thymidine label (cpm) incorporated in triplicate cultures.

TABLE 8A

GAD Induced Tolerance

Treatment	Insulitis Score[a]	N	Spleen Cell Proliferation (SI ± SEM)[b]						
			β-Gal	GAD	GAD Peptides #17	GAD Peptides #34	GAD Peptides #35	hsp Peptide	CPH
Uninjected	2.4 ± 0.2	5	1.0 ± 0.2	9.5 ± 2.1	4.8 ± 0.4	6.0 ± 0.1	2.9 ± 0.2	6.7 ± 1.0	ND[c]
β-Gal.	2.6 ± 0.6	5	1.1 ± 0.1	15.4 ± 1.8	5.1 ± 0.6	5.1 ± 0.6	4.0 ± 0.2	6.6 ± 0.5	11.5 ± 0.9
GAD 1.1 ± 0.02	0.1 ± 0.1	8	1.1 ± 0.03	1.6 ± 0.3	1.0 ± 0.05	1.2 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	
hsp-p	1.7 ± 0.4	5	1.1 ± 0.05	5.8 ± 0.2	4.5 ± 0.1	4.1 ± 0.3	4.2 ± 0.1	1.1 ± 0.04	4.4 ± 0.2
m-hsp	1.8 ± 0.5	5	1.0 ± 0.1	4.2 ± 0.1	3.9 ± 0.1	3.9 ± 0.1	3.4 ± 0.2	1.0 ± 0.03	4.3 ± 0.2

[a] Severity of mononuclear cell infiltration was defined histologically (0 = no lymphocytic infiltration; 1 = (25%; 2 = 25-50%; 3 = 50-75%; 4 =)75%) (Qin, *et al., Immunol.*, 150:2072-2080, 1993). Score is mean ± SE.

[b] Significant responses noted by solid underline, borderline responses noted by double underline.

[c] Not determined.

TABLE 8B

GAD Induced Tolerance

Treatment	Insulitis Score[a]	N	Spleen Cell Proliferation (SI ± SEM)[b]						
			β-Gal	GAD	GAD Peptides			hsp Peptide	CPH
					#17	#34	#35		
11 peptide	~2.5		1.0 ± 0.1	21.1 ± 2.2	13.7 ± 1.5	11.4 ± 1.5	11.3 ± 0.7	13.3 ± 0.9	ND
34/35 peptides (+IFA)	0.7 ± 0.4		1.0 ± 0.2	1.9 ± 1.1	2.2 ± 1.2	1.1 ± 0.3	1.0 ± 0.1	1.8 ± 1.1	ND
IFA alone (+IFA)	~2.5		1.0 ± 0.1	8.1 ± 0.5	5.0 ± 0.5	4.8 ± 0.5	4.8 ± 0.4	6.6 ± 0.6	ND

[a] Severity of mononuclear cell infiltration was defined histologically (0 = no lymphocytic infiltration; 1 = (25%; 2 = 25-50%; 3 = 50-75%; 4 =)75%) (Qin, *et al., Immunol.*, 150:2072-2080, 1993). Score is mean ± SE.

[b] Significant responses noted by solid underline, borderline responses noted by double underline.

[c] Not determined.

TABLE 8C

GAD Induced Tolerance

Treatment	Insulitis Score[a]	N	Spleen Cell Proliferation (SI ± SEM)[b]						
			β-Gal	GAD	GAD Peptides			hsp Peptide	CPH
					#17	#34	#35		
11 peptide	≈(?)		1.4 ± 0.4	12.1 ± 3.5	6.9 ± 0.4	6.4 ± 1.0	6.8 ± 0.3	8.7 ± 0.9	ND
HEL peptide	(?)		1.2 ± 0.2	10.7 ± 3.5	6.4 ± 1.4	5.9 ± 1.6	6.9 ± 1.9	8.0 ± 1.8	ND
34/35 peptides (+IFA)	(?)		1.0 ± 0.1	4.4 ± 2.6	1.8 ± 1.1	1.0 ± 0.1	1.1 ± 0.2	2.7 ± 1.3	ND

[a] Severity of mononuclear cell infiltration was defined histologically (0 = no lymphocytic infiltration; 1 = ⟨25%; 2 = 25-50%; 3 = 50-75%; 4 = ⟩75%) (Qin, *et al., Immunol.*, 150:2072-2080, 1993). Score is mean ± SE.

[b] Significant responses noted by solid underline, borderline responses noted by double underline.

[c] Not determined.

Seventy five percent of the GAD treated mice, but none of the controls, showed no T-cell reactivity to GAD (indicating complete tolerization) or to other βCAs. These mice were also completely free of insulitis (score 0.0). If there were another effector T cell population in the islets, specific for an unknown βCA, that preceded the anti-GAD response, the release of cytokines by this population should have promoted T-cell responses to βCAs and insulitis (Sarvetnick, *et al.*, *Nature*, 346:844, 1990; Heath, *et al.*, *Nature*, 359:547, 1992). Twenty five percent of the GAD-treated mice were not completely tolerized to GAD, as evidenced by a weak residual GAD reactivity (SI of about 3) and displayed very limited peri-insulitis. In contrast, while tolerization to both of the hsp antigens was complete, these treatments reduced, but did not prevent, the development of T cell responses to other βCAs or insulitis. Thus, while the inactivation of GAD-reactive T cells prevented β cell autoimmunity, hsp tolerization only partially reduced it, as would be expected if a secondary element was removed from the amplifactory cascade.

In ongoing experiments examining the effects of GAD tolerization on diabetes incidence, all of the GAD treated mice (n=17, presently 37 weeks old) have normal glucose levels, while 70% of the mice receiving control antigens developed hyperglycemia by 19 weeks of age (n=20). Five GAD treated mice were sacrificed at 30 weeks of age. All were free of detectable βCA reactive T cells. Of these five animals, four mice were completely free of insulitis and one mouse displayed very limited peri-insulitis. These data show that inactivation of GAD reactive T-cells prevents the long term development of insulitis and diabetes.

2. In a sec nd set of experiments neonatal female NOD mice were injected intraperitoneally IFA with peptide 11, a mixture of peptides 34 and 35 plus IFA,

or with IFA alone and at 12 weeks of age the mice were examined for insulitis and autoantigen reactive T-cells as in Example 6.1. Proliferative splenic T-cell responses induced by the various antigens were performed as in Example 4, and data in Table 8B are expressed as the average [^{3}H]-thymidine label (cpm) incorporated in triplicate cultures.

The data in Table 8B show that tolerization with control peptide 11 did not prevent auto antibody response to GAD or to GAD peptides 17, 34 or 35. Nor was response to hsp peptide prevented by tolerization with peptide 11. IFA alone was somewhat effective at suppressing immune response. By contrast, tolerization with the mixture of peptides 34 and 35 suppressed the autoimmune response of spleen cell proliferation to all species tested: β-gal, GAD, GAD peptides 17, 34 and 35, and hsp peptide. In addition, tolerization to GAD peptides 34 and 35 greatly reduces insulitis but does not completely prevent it as whole GAD65 does.

3. In a third set of experiments female NOD mice were intravenously injected at three weeks of age with peptide 11 (control), a mixture of peptides 34 and 35 plus IFA, or with HEL peptides and at 12 weeks of age the mice were examined for insulitis and autoantigen reactive T-cells as in Example 6.1. Proliferative splenic T-cell responses induced by the various antigens were performed as in Example 4, and data in Table 8C are expressed as the average [^{3}H]-thymidine label (cpm) incorporated in triplicate cultures.

The data in Table 8C show that tolerization with control peptide 11 did not prevent auto antibody response to GAD, to GAD peptides 17, 34 or 35 or to hsp although the response was not as great as in Example 6.2. Nor was response to hsp peptid pr vented by immunization with peptide 11. By contrast, immunization with the mixture of peptides 34 and 35 plus IFA suppressed the autoimmune response of spleen

cell proliferation to all species tested: β-gal, GAD, GAD peptides 17, 34 and 35, and hsp peptid .

EXAMPLE 7

CHARACTERIZATION OF GAD-REACTIVE T-CELLS

This example describes studies on GAD-Reactive T-Cells for additional properties that distinguish activated/memory from resting/naive lymphocytes.

In one series of experiments γ interferon (IFNγ) was measured by ELISA in culture supernatants (CSN) of spleen cells of 6-9 week old mice after challenge with GAD or control antigens HEL and MBP. Additionally, the frequency of antigen specific, IFNγ-producing cells was determined by an ELISA spot technique (T. Taguchi, *et al.*, *J. Immunol.*, 145:68-77, 1990). Frequency of antigen-induced, spot forming cells (SFC) among 10^3 spleen cells is represented in Figure 9(a). Values are the mean + SEM from 5 individual female NOD mice, each tested in triplicate cultures with or without antigen. Results from a single experiment are shown. These are representative of 3 separate experiments.

In performing these experiments, freshly isolated spleen cells were cultured with or without antigen as described in Example 4. CSN were taken after 48 h and the concentration of IFNγ was determined by ELISA (Macy, *et al.*, *FASEB J.*, 3003-3009, 1988). IFNγ specific monoclonal antibody (mAb) R4-6A2 (Pharmingen) was used as the capturing reagent and biotinylated mAb XMG 1.2 (Pharmingen, also specific for IFNγ) was used in conjunction with streptavidin-alkaline phosphatase (Zymed) and p-nitrophenol for detection of bound lymphokine. Recombinant murine IFNγ (Pharmingen) was used as a standard. ELISA spot assays for the detection of antigen-specific, IFNγ-producing cells w re performed as described (Taguchi, *et al.*, *J. Immunol.*, 145:68-77, 1990). After a 24h pre-activation culture of spleen cells with our without antigen, cells were transferred by serial dilution to 96 well

microtiter plates (Millipore) that had been pre-coated with mAb R4-6A2. After 24h, the cells were removed and IFNγ spots were visualized using XMG 1.2-biotin in conjunction with nitroblue terazolium-bromochloroindolyl phosphate substrate (Sigma). Spots were counted visually and the frequency of antigen specific cells was determined from the difference between the number of spots seen with and without antigen.

As shown in Figure 9(a), when freshly isolated T-cells from 6-9 week old NOD mice were challenged with GAD or control antigens, high concentrations of IFNγ were detected only in cultures containing GAD, suggesting that the GAD specific T-cells had been pre-activated *in vivo*, since only pre-activated T-cells (Th1) produce IFNγ within 48 hours after antigen recognition (Ehlers, *et al.*, *J. Exp. Med.*, 173:25-36 1991; Croft, *et al.*, *J. Exp. Med.*, 176:1431-1437, 1992). In contrast, T-cells from age matched BALB/c mice did not respond to GAD or to control antigens by IFNγ production (data not shown).

Results of the ELISA spot assay to measure directly the frequency of GAD-specific T-cells showed that while in 6-9 week old NOD mice, T-cells reactive to control antigens constituted approximately 1 in 10^5 cells in the spleen, the frequency of GAD-reactive T-cells was about two orders of magnitude higher, ranging from 90-291 cells per 10^5 cells (Figure 9(a), confirming the data obtained by proliferation assays (Figure 8) that these cells had been clonally expanded *in vivo*.

In another series of experiments, GAD specific T-cells were characterized for expression of the cell surface marker L-selectin, since murine T-cells convert from an L-selectin$^+$ (L-sel$^+$) to an L-selectin$^-$ (L-sel$^-$) phenotype upon activation (Bradley, *et al.*, *J. Immunol.*, 148:324-331, 1992).

To perform these studies, pooled spleen cells from 3 to 4 ag matched mice were panned on plates coated with goat-anti-mouse Ig (Zymed) to remove adherent macrophages as well as B cells. Next, CD8+ cells were coated with mAb 58.6-72 (ATCC) and removed by panning over plates coated with goat-anti-rat Ig (Zymed). The non-adherent CD4+ cell fraction was labeled with anti-L-selectin mAb MEL-14 (ATCC) and panned on goat-anti-rat Ig coated plates. Both the adherent (CD4+ L-sel$^+$) and non-adherent (CD4+,L-sel$^-$) fractions were sampled. Purity of the cell fraction was assessed by FACS analysis; cells were)90% CD4+ and)95% enriched for the L-sel$^-$ or L-sel$^+$ phenotype. The purified cell fractions were tested for GAD reactivity by seeding them at 2 x 10^5 cells per well in 96 well microtiter plates with or without antigen. Irradiated (3000 rad), unseparated spleen cells of 3 week old NOD mice were added at 5 x 10^5 cells per well as a source of antigen presenting cells. Supernatants of triplicate cultures were taken 48h later and their IFNγ content was determined by ELISA.

The results of this study showed that by 2-3 weeks of age, GAD reactive T-cells could not be detected in either the L-sel$^+$ or the L-sel$^-$ population, consistent with a low frequency of antigen reactive precursors at this time point. However, by 6 weeks of age high levels of IFNγ were induced by GAD (but not by control antigens) in the L-sel$^-$ (but not the L-sel$^+$) subpopulation of CD4+ cells (Figure 9(b)).

The increase in clonal size of GAD reactive T-cells, their production of IFNγ and their L-sel$^-$ phenotype provide three independent lines of evidence that a potentially pathogenic (Ando, *et al. Cell Immunol.*, 124:132-143, 1989) Th1 type T-cell respons is sp ntan ously primed to GAD *in vivo* early in NOD development.

EXAMPLE 8

CHARACTERIZATION OF AD SPECIFIC T-CELL DETERMINANT RECOGNITION

The fine specificity of the anti-GAD T-cell response was mapped using a set of 38 peptides (numbered successively from the N-terminus) that were 20-23 amino acids (aa) long and span the entire GAD_{65} (Bu, *et al.*, *Proc. Natl. Acad. Sci.*, 89:2115-2119, 1992) sequence with 5aa overlaps (Figure 10).

Spleen cells were tested from 4 (Figure 10a), 5 (Figure 10b) and 7 (Figure 10c) week old NOD mice for proliferative responses (as described in Example 4) to the GAD peptides. Peptides were present in cultures at 7 μM and the label was added during the last 16 hours of a 5-day culture. The peptides were synthesized using standard Fmoc chemistry and purified by reverse phase HPLC (Advanced Chemtech). The sequence of stimulatory peptides are shown below in Table 9.

TABLE 9

Peptide Number	GAD Region	Amino Acid Sequence
6	78-97	KPCSCSKVDVNYAFLHATDL
17	247-266	NMYAMMIARFKMFPEVKEKG
23	335-356	TAGTTVYGAFDPLLAVADICKK
32	479-498	EYLYNIIKNREGYEMVFDGK
34	509-528	IPPSLRYLEDNEERMSRLSK
35	524-543	SRLSKVAPVIKARMMEYGTT
36	539-558	EYGTTMVSYQPLGDKVNFFR
38	566-585	ATHQDIDFLIEEIERLGQDL

Murine and human GAD_{65} are 95% identical at the amino acid level (555/585) and are 98% conserved, with most of the differences localized near their N-termini. The underlined amino acid in the stimulatory peptide

sequences above are conservatively substituted in murine GAD_{65}. In separate experiments, the murine form of key peptides (#17 and #34) were tested and produced similar results.

As shown in Figure 10, peptides that triggered stimulation indices $\rangle 3$ are indicated as black bars. These peptides did not induce proliferation in T-cells from NOD mice $\langle 3$ or $\rangle 16$ weeks in age, or from control (BALB/c x NOD)F1 mice (data not shown). The data are represented as the mean SI ± standard error calculated from 3-6 individual mice tested twice in each age group. Characteristic results for peptide induced blastogenesis in individual mice are shown in Table 6. The first detectable response, at 4 weeks of age, was confined to the carboxy-terminal region of GAD, and involved two adjacent peptides (aa 509-528 and 524-543, peptides #34 and #35, respectively, Figure 10a). At 5 weeks of age, responses to an additional determinant (aa 247-266, peptide #17, which contains a region of sequence similarity with Coxsackievirus (Kaufman, *et al., J. Clin. Invest.*, 89:283-292, 1992) (Figure 10b) were regularly recorded. During the next two weeks, responses to peptide #17 (aa247-266) increased and T-cell autoimmunity spread to two additional peptides at the carboxy terminus (aa 479-498 and 539-558; peptides #32 and #36 respectively, Figure 10c). Subsequently, reactivity to the GAD peptides declined (data not shown), paralleling the loss of response to the whole protein (Figure 8). It is unclear why the initial T-cell response to βCAs fades in NOD mice. Possible explanations include: a) immune regulatory mechanisms; b) exhaustion of the response due to the continuous stimulation by the endogenous antigen; and c) induction of anergy in specific T-cells owing to their recognition of the autoantigen on "non professional" antigen presenting cells such as the β cells themselves (Markmann, *et al., Nature*, 336:476-479, 1988).

The gradual diversification of the primed autoreactive T-cell repertoire that was observed in this naturally occurring autoimmune disease parallels the shifts in T-cell recognition recently observed in experimentally induced autoimmunity to the CNS where autoreactivity spreads both intra- and intermolecularly among CNS proteins (Lehmannn, *et al.*, *Nature*, 358:155-157, 1992; Perry, *et al.*, *J. Neuroimmunol.*, 33:7-15, 1991; Watanabe, *et al.*,*Nature*, 305:150-153, 1983; Liebert, *et al.*, *J. Neuroimmunol.*,17:103-118, 1988). Apparently, lymphokine secretion by the first wave of autoantigen specific T-cells in the target organ results in up-regulation of antigen presentation and creates a microenvironment that favors priming of additional autoreactive T-cells (Lehmann, *et al.*, *Immunol. Today*, 14:203-208, 1993; Sarvetnick, *et al.*, *Nature*, 346:844-847, 1990; Heath, *et al.*, *Nature*, 359:547-549, 1992). Since hsp reactive CD4+ T-cells are capable of inducing IDDM (Elias, *et al.*, *Proc. Natl. Acad. Sci.*, 87:1576-1580, 1990; Elias, *et al.*, *Proc. Natl. Acad. Sci.*, 88:3088-3091, 1991), their recruitment into the activated T-cell pool, along with T-cells reactive to other βCAs, probably reflects an amplificatory cascade that eventually leads to β cell destruction.

In summary, the data above establish GAD as a critical target antigen in the pathogenesis of IDDM in NOD mice. The results show that T-cell responses to βCAs diversify both intramolecularly and intermolecularly as the disease progresses, consistent with a dynamic autoimmune repertoire (Lehmann, *et al.*, *Immunol. Today*, 14:203-208, 1993). However, interference with the early autoreactive T-cell population can prevent the recruitment of additional autoantigens into the primed repertoire thereby halting a cascade of autoimmune respons s that eventually l ads to β cell destruction. As a similar autoimmune

progression is also likely to occur during the development of human IDDM (Palmer, J.P., Predicting IDDM, *Diabetes Reviews*, 1:14-115, 1993; Atkinson, *et al.*, *Lancet*, 339:458-459, 1992), these findings suggest that peptide-based immunotherapeutic agents would be useful in predicting and ameliorating human IDDM.

EXAMPLE 9

AUTOANTIBODY REACTIVITY WITH GAD FRAGMENTS

This example describes a study which examined the variability in recognition of epitopes in human GAD_{65} polypeptides by IDDM autoantibodies in sera of human patients.

Portions of human GAD_{65} cDNA were amplified by the polymerase chain reaction (PCR; Saiki, *et al.*, *Science*, 239:487, 1988) to produce DNA segments encoding three polypeptide segments: amino acid residues 1-224 (segment A); 224-398 (segment B); and 398-585 (segment C). Each construct also contained a T_7 promoter, a consensus sequence for the initiation of translation and an initiating methionine codon (Korak, M., *J. Cell Biol.*, 108:229, 1989). Each PCR product was then trascribed *in vitro* with T_7 RNA polymerase and translated *in vitro* in a rabbit reticulocyte cell-free system in the presence of ^{35}S-methionine, using conditions recommended by the supplier (Amersham Corp., Arlington Heights, IL). Each test serum (30 μl) was incubated with the resulting ^{35}S labeled-polypeptides. The bound peptides were isolated with PAS and analyzed by SDS-PAGE in 12% polyaacrylamide and autoradiography.

TABLE 10

IDDM PATIENT SERA REACTIVITY WITH GAD SEGMENTS

	SEGMENT			
PATIENT	A	B	C	
Control (N=7)	-	-		-
052	-	+		+
723	-	-		-
705	-	+		+
UC2	-	+	+	
N.L.	-	-		-
L.I.	-	-		-
T.L.	-	-		-
P.T.	-	+		-
J.D.	-	-		-
B.Y.	-	+		+
M.C.	-	-	-	
R.S.	-	-		-
K.O.	-	-	-	
T.B.	-	-		-
S.M.	-	-	-	
A.W.	-	+	-	
J.B.	-	+		+
J.A.	-	-		-
P.C.	-	+		+
L.R.	-	-		-
J.M.	-	+		-
G.A.	-	-	-	

As shown in Table 10, none of the sp cimens had detectable levels of antibodies to the amino terminal third (segment A) of GAD whereas 9 patients (41%) had antibodies reactive with the middle third (segment B) and 6 patients (27%) had antibodies to the carboxyl-terminal third (segment C) of GAD.

EXAMPLE 10

PREDICTION OF INCIPIENT IDDM BY GAD EPITOPE RECOGNITION PATTERN

The increasing likelihood of an IDDM interventive therapy and the (recently acknowledged) benefits of managed glucose homeostasis in preventing IDDM associated complications makes the early detection of β cell autoimmunity before clinical IDDM onset and in NIDDM patients (10% of whom eventually convert to IDDM) a crucial goal. Autoanitbodies to GAD may provide the earliest and most reliable marker of impending IDDM among the molecularly defined IDDM associated autoantigens. To determine whether GAD peptides will bind to IDDM associated autoantibodies the following study was conducted.

A set of peptides (20-23 amino acids in length, with 5 aa overlaps) that span the human GAD65 molecule were synthesized to determine whether sera from most individuals at risk, pre-IDDM and with IDDM (in contrast to healthy controls) do in fact produce antibodies that differentially recognize GAD65 linear epitopes distributed throughout the molecule.

Patient sera and most control sera were those used in a previous study (Kaufman, *et al.*, J. Clin. Investigation, *supra*) All samples were coded and tested in a blind manner. Peptides were synthesized using an automatic instrument (Applied Biosystems, Foster City, CA) and standard conditions. Peptides were dissolved in 60 mM sodium bicarbonate buffer (pH 9.6) at 20 ug/ml and 100 ul of each was add d to duplicated wells of a 96 well Nunc-Immuno Plate. P ptides w r allowed t

bind at 4°C overnight. The plates were then washed three times with PBS + 0.1% Tween 20 (wash buffer), after which the plates were pre-absorbed with 3% BSA in sodium bicarbonate buffer for 0.5 hours at 37°C, or at room temperature overnight. The plates were then washed 5 times with the above wash buffer. 100 ul of serum at a 1/300 dilution in PBS + 0.1% Tween 20 and 1% BSA was added to each well and antibodies were allowed to bind for 1 hour at 37°C. The plates were washed 5 times with wash buffer. 100 ul of a 1/600 dilution of HRP-goat anti-human IgG (BRL, Gaithersberg, MD) was added to each well and allowed to bind for 1 hour at 37°C. The plates were then washed 7 times and 100 ul of substrate buffer was added to each well for 30 minutes at room temperature. The color development was measured at 410 nm using an ELISA plate reader (ICN, Biomedicals, Costa Mesa, CA). Positive sera were defined as: OD_{410} of the sample/negative control ≥3.0.

The data shown in Table 11 establish that a number of GAD peptides were recognized by patients previously shown to be 64K positive, but not by control sera. Each patient showed a different pattern of GAD epitope recognition. Peptides 20, 21 and 25, were each recognized by 6/8 patients, and none of the controls--with the exception of peptide 25 which was recognized by 1 out of 13 controls. Based on immunoreactivity to 2 of these peptides (#20 and 21) 7/8 (88%) of the patients (and none of the controls) could be identified as possessing GAD autoantibodies. Peptides 3, 6, 22, 25 and 37 were each recognized by only 25-37% of the patients (and none of the control sera), but taken together, 75% of the patients recognized at least one of thes . Peptides 5, 9 and 24 were oft n positiv for immunor activity by both control and patient sera.

This level of sensitivity is comparable to the best currently available assays using whole GAD65 purified

from brain or recombinant organisms. Besides avoiding laborious antigen purification, peptide based autoantibody screening, together with PCR based HLA typing, may reveal epitope recognition patterns associated with progression or lack of progression to IDDM and its associated complications. Individuals determined to be at high risk could then consider therapeutic intervention.

It should also be noted that the GAD peptides recognized by autoantibodies were different from those recognized by NOD GAD reactive T cells in Example 6.

TABLE 11

EPITOPE RECOGNIATION OF HUMAN GAD65 PEPTIDES

PEPTIDE

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Controls																																						
A.P.					X				X															X														
P.T.					X																			X														
5748					X				b															X														
5620					X				b															X		b				b								
4380					X				b															X								b						
S.M.					X				X												b			X	X		b											
FA8																								X														
FA12																								X	b													
1688					b																			X														
FA9					X																			X														
H.B					b																			X														
FA2					X																			X														
FA11					X																			X														
									2																1													
IDDM																																						
At Risk																																						
052		b			X	X			b					b	b	b			X	X				X				b	X		b							b
825					X						X													X	b													
356					X				X												X			X	X													
L.I.			b		X			X			b									b				X								b						

	PEPTIDE																																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
<u>Pre-IDDM</u>																																						
J.A.					X																X			X														
723			X		X				X											X	X			X	X										X		X	
P.T. (1)			X		X				b											X	b			X	b				X			b						
(2)			b		X		b	b	b											X				X	X				X			b		b				
J.B. (1)					b		b				b													X														
(2)			b		X		b	b			b									b	b			X	b				b									
R.S. (1)			X		X				b		b													X	X													
(2)			X		X	b		b	b		b									b				X	X													
B.Y. (1)					X						X													X														
(2)					X																			X														
J.B. (1)					b		b				b													X														
(2)			b		X		b	b			b									b	b			X	b				b									
<u>At Onset</u>																																						
705					X			X												X	X	X		X	X				X							X	X	
S.H.		b	X		X															X	X			X	X													b
291			b	X	X		b													X	X	X		X	X		X					b		b				
048			X		X	X			X											X	X			X	X				X			X		X				X
			5	1		2		2	3		2								1	7	7	2			8		1		4			1		1	1	1	2	1

X = positive for immunoreactivity as defined by OD/background≥3.0
b = borderline response (OD/background =2.5-2.9).

EXAMPLE 11

GAD IMMUNIZATION PROTECTS NOD MICE FROM IDDM

The availability of cDNAs encoding GAD65 allows the testing of this molecule in new interventive therapies designed to interfere with GAD-specific T cells. Tests were conducted to examine the ability of GAD65 immunization to protect NOD mice at 8 weeks of age, a time at which T cell responses to a number of b cell antigens and insulitis is already well established. If GAD immunotherapy was effective at this stage, it would hold promise for treatment in humans in which the autoimmune process has already been established.

METHODS

Antigens

An IPTG inducible T7 expression vector was used to express both human GAD65 and *E. coli* b galactosidase (β-gal). In IPTG induced recombinant *E. coli*, GAD and β-gal constitute about 10-20% of the total bacterial protein. However, almost all of the GAD was in inclusion bodies, which could be isolated and extensively washed to obtain material that is about 80% GAD. We then did affinity purifications of GAD and β-gal on the basis of a hexa-histidine "tag" which was attached to GAD during the subcloning process. These extra histidine residues allow the rapid affinity purification (Novagen) of GAD by metal affinity chromatography (Hochuli, *et al.*, Bio Technology, 6:1321-1325, 1988). The inclusion body material is solubilized in 6M guanidine hydrochloride (GHCL), 10mm β-mercaptoethanol and 1% triton X-100. After binding to the column, the column was extensively washed with GHCL and 8M urea in phosphate buffers. Only the central peak GAD fraction was utilized for subsequent studies. Human GAD65 shares 96% amino acid sequence identity with murine GAD65, with most of the amino acid differences being conservative substitutions.

The GAD preparation appeared t be free of immunologically detectable contaminants. It also appeared to be free of bacterial contaminants on overloaded silver stained gels. Analysis by a national reference laboratory found ⟨0.06ng LPS/ug GAD. Human GAD65 did not induce T cell proliferation in ⟨4 or ⟩16 week old NOD or control BALB/c or (NOD/BALB/c) F1 spleen cells. The results using synthetic GAD peptides (Figure 10) precisely parallel the data using whole recombinant GAD (Figure 8). Other antigens described herein elsewhere that are not involved in IDDM (such as the beta galactosidase) did not induce NOD T cell responses. After immunizing mice with GAD, we were unable to detect cross reactive T cell responses in recall experiments with other proteins that were purified from recombinant *E. coli* by the same metal affinity chromatography procedure. Amino acid sequence analysis of GAD and β-gal each gave a single expected amino acid N-terminal sequence. If there had been appreciable endotoxins, heat shock proteins, or other contaminants present in the GAD preparation, spleen, PBMC (Atkinson, *et al.*), and T cell proliferation responses that were not disease specific would have been expected.

Breeder mice were purchased from Taconic Farms and housed under specific pathogen-free conditions. Only female NOD mice were used in this study. The average age of IDDM onset in unrelated females in the colony was 22 weeks. Insulitis is generally observed beginning at 4 weeks of age. T cell responses to GAD, HSP, CPH were found by 6 weeks in age. The incidence of IDDM in female mice is 70-90% by one year of age.

Immunizations

At 8 weeks of age, 25 ug GAD or control β-gal. was inject d intraperitoneally (ip) in 100 ul of incomplet Freunds adjuvant (IFA). Because there may be a requirement for continual antigen presentation

(Ramsd 11, *t al.*, Science, 257:1130-1133, 1992) mice were treated again every 6 weeks. Urine glucose levels were monitored twice weekly. After observing above normal glucose in urea, blood glucose levels were monitored twice weekly. Two consecutive blood glucose level readings of 300 mg/ml was considered as IDDM onset, after which the mice were sacrificed and spleen cells were tested as described above in Example 6 for evidence of spleen cell proliferation.

Immunization of 8 week old NOD mice produced a clear delay in the onset of IDDM compared to control β-gal immunized mice (Fig. 11). While two of the GAD immunized mice (open circles) developed IDDM at about the normal age of onset (20 weeks), the other 8 GAD immunized mice showed no signs of hyperglycemia until 36 weeks in age. Four of the GAD treated mice developed IDDM between 37 and 40 weeks in age. Four of the GAD treated mice currently remain disease free (at 52 weeks of age). In contrast, the majority of β-gal injected mice (closed circles) had hyperglycemia by 22 weeks of age and 6/10 developed IDDM by 27 weeks in age. At 52 weeks of age, 2 of the β-gal treated mice remain disease free. This experiment shows that GAD immunization significantly delayed (<0.02) or prevented diabetes of NOD mice in which β cell autoimmunity has already significantly progressed.

β cell autoimmunity is already well established at 8 weeks of age, and it is likely to also be in individuals determined to be at risk for IDDM on the basis of circulating autoantibodies. Although the mechanism of this protection is not clear, periodic injections of GAD have a profound moderating effect on the induction of disease.

TABLE 11

AMINO ACID SEQUENCES FOR GAD65

1	MASPGSGFWSFGSEDGSGDS
2	GSGDSENPGTARAWCQVAQKFTG
3	QKFTGGIGIGIGNKLCALLYGD
4	LLYGDAEKPAESGGSQPPRA
5	QPPRAAARKAACACDQKPCSC
6	KPCSCSKVDVNYAFLHATDL
7	HATDLLPACDGERPTLAFLQ
8	LAFLQDVMNILLQYVVKSFDRS
9	SFDRSTKVIDFHYPNELLQE
10	ELLQEYNWELADQPQNLEEILM
11	EEILMHCQTTLKYAIKTGHP
12	KYGHPRYFNQLSTGLDMVGL
13	DMVGLAADWLTSTANTNMFT
14	TNMFTYEIAPVFVLLEYVTL
15	EYVTLKKMREIIGWPGGSGD
16	GGSGDGIFSPGGAISNMYAM
17	NMYAMMIARFKMFPEVKEKG
18	PEVKEKGMAALPRLIAFTSE
19	AFTSEHSHFSLKKGAAALGI
20	AALGIGTDSVILIKCDERGK
21	DERGKMIPSDLERRILEAKQ
22	LEAKQKGFVPFLVSATAGTT
23	TAGTTVYGAFDPLLAVADICKK
24	DICKKYKIWMHVDAAWGGGLLMS
25	GLLMSRKHKWKLSGVERANS
26	ERANSVTWNPHKMMGVPLQC
27	VPLQCSALLVREEGLMQNCNQ
28	QNCNQMHASYLFQQDKHYDL
29	KHYDLSYDTGDKALQCGRHV
30	CGRHVDVFKLWLMWRAKGTTG
31	KGTTGFEAHVDKCLELAEYLYN
32	EYLYNIIKNREGYEMVFDGK
33	VFDGKPQHTNVCFWYIPPSL
34	IPPSLRTLEDNEERMSRLSK

35 SRLSKVAPVIKARMMEYGTT

36 EYGTTTMVSYQPLGDKVNFFR

37 VNFFRMVISNPAATHQDIDF

38 ATHQDIDFLIEEIERLGQDL

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made without departing from the scope of the invention.

CLAIMS

1. A polypeptide consisting essentially of an amino acid sequence selected from the group consisting of:

KPCSCSKVDVNYAFLHATDL;
NMYAMMIARFKMFPEVKEKG;
TAGTTVYGAFDPLLAVADICKK;
EYLYNIIKNREGYEMVFDGK;
IPPSLRYLEDNEERMSRLSK;
SRLSKVAPVIKARMMEYGTT;
EYGTTMVSYQPLGDKVNFFR;
ATHQDIDFLIEEIERLGQDL;
LAFLQDVMNILLQYVVKSFDRS;
EEILMHCQTTLKYAIKTGHP;
DERGKMIPSKLERRILEAKQ;
KHYDLSYDTGDKALQCGRHV;
AALGIGTDSVILIKCDERGK;
GLLMSRKHKWKLSGVERANS;
LEAKQKGFVPFLVSATAGTT; and
VNFFRMVISNPAATHQDIDF.

2. An isolated polynucleotide sequence encoding a polypeptide of claim 1.

3. The polynucleotide of claim 2 which is DNA.

4. A method for detecting autoantibody to GAD in a patient specimen which comprises contacting the specimen with a GAD fragment and determining whether autoantibody binds to the fragment.

5. The method of claim 4, wherein the fragment is a polypeptide of at least about 16 amino acids in length and is selected from the amino acid sequence from about amino acid 224 to about amino acid 585 of GAD_{65}.

6. The method of claim 4, wherein the fragment is a polypeptide of claim 1.

7. Th m thod of claim 4, wherein the specimen is blood.

8. The method of claim 4, wherein the peptide is detectably labeled.

9. The method of claim 8, wherein the detectable label is selected from the group consisting of a radioisotope, a fluorescent compound, a colloidal metal, a chemiluminescent compound, a bioluminescent compound, a phosphorescent compound, and an enzyme.

10. The method of claim 4, wherein the peptide is bound to a solid phase.

11. A monoclonal antibody to the peptide of claim 1.

12. A hybridoma cell line capable of producing the monoclonal antibody of claim 9.

13. A vector containing the polynucleotide of claim 2.

14. A host cell transformed with the polynucleotide of claim 2.

15. A method for ameliorating a GAD associated autoimmune disorder in a patient having or at risk of having the disorder, which comprises administering to the patient a therapeutically effective amount of whole GAD_{65} or a therapeutically effective fragment thereof.

16. The method of claim 15, wherein the therapeutically effective fragment is a polypeptide of at least about 16 amino acids in length and is selected from the amino acid sequence from about amino acid 224 to about amino acid 585 of GAD_{65}.

17. The method of claim 15, wherein the therapeutically effective fragment is selected from the group consisting of:

KPCSCSKVDVNYAFLHATDL;
NMYAMMIARFKMFPEVKEKG;
TAGTTVYGAFDPLLAVADICKK;
EYLYNIIKNREGYEMVFDGK;
IPPSLRYLEDNEERMSRLSK;
SRLSKVAPVIKARMMEYGTT;
EYGTTMVSYQPLGDKVNFFR; and
ATHQDIDFLIEEIERLGQDL.

18. The method of claim 15, wherein the therapeutically effective fragment is selected from the group consisting of:

KPCSCSKVDVNYAFLHATDL;
LAFLQDVMNILLQYVVKSFDRS;
EEILMHCQTTLKYAIKTGHP;
DERGKMIPSKLERRILEAKQ;
KHYDLSYDTGDKALQCGRHV;
AALGIGTDSVILIKCDERGK;
GLLMSRKHKWKLSGVERANS;
LEAKQKGFVPFLVSATAGTT; and
VNFFRMVISNPAATHQDIDF.

19. The method of claim 16, wherein the disorder is IDDM.

20. The method of claim 16, wherein the disorder is stiff man disease.

21. The method of claim 16, wherein the administration is enteral.

22. The method of claim 21, wherein the enteral administration is oral.

23. The method of claim 15 or 16, wherein the administration is parenteral.

24. The method of claim 23, wherein the parenteral administration is by subcutan ous, intramuscular, intraperitoneal, intracavity, transdermal, intranasal, or intravenous inj ction.

25. The m thod of claim 15, wherein said administration is at a dosage of about 0.01 mg/kg/dose to about 2000 mg/kg/dose.

26. The method of claim 15, wherein the polypeptide is therapeutically labeled.

27. The method of claim 26, wherein the therapeutic label is selected from the group consisting of a radioisotope, a drug, a lectin, and a toxin.

28. A method for detecting the status of a GAD associated autoimmune disorder in a patient which comprises contacting a T-cell of the patient with at least one polypeptide of claim 1 and detecting the response of the T-cell to the peptide.

29. The method of claim 26, wherein the response of the T-cell is stimulation.

30. The method of claim 27, wherein the stimulation is measured by cellular uptake of radiolabelled nucleoside.

31. The method of claim 30, wherein the nucleoside is ^{3}H thymidine.

32. A kit useful for the detection of antibody to the polypeptide of claim 1 in a specimen suspected of containing such antibody, the kit comprising carrier means compartmentalized to receive in close confinement therein one or more containers comprising a container containing the polypeptide of claim 1.

33. A kit useful for determining the status of a GAD associated disorder in a specimen of a patient, the kit comprising carrier means compartmentalized to receive in close confinement therein one or more containers comprising a container containing a polypeptid of claim 1.

34. A method for detecting a GAD associated autoimmune disorder in a patient having or at risk of having the disorder, which comprises administering to the patient a diagnostically effective amount of a diagnostically effective fragment of GAD_{65}.

35. The method of claim 34, wherein the diagnostically effective fragment is a polypeptide of at least about 16 amino acids in length and is selected from the amino acid sequence from about amino acid 34 to 573 of GAD_{65}.

36. The method of claim 35, wherein the diagnostically effective fragment is selected from the group consisting of:

KPCSCSKVDVNYAFLHATDL;
LAFLQDVMNILLQYVVKSFDRS;
EEILMHCQTTLKYAIKTGHP;
DERGKMIPSKLERRILEAKQ;
KHYDLSYDTGDKALQCGRHV;
AALGIGTDSVILIKCDERGK;
GLLMSRKHKWKLSGVERANS;
LEAKQKGFVPFLVSATAGTT; and
VNFFRMVISNPAATHQDIDF.

37. The method of claim 35, wherein the disorder is IDDM.

38. The method of claim 35, wherein the disorder is stiff man disease.

feline (from cDNA)
YEIAPVFVLMEQITLKKMREIVGWSSKDGDGIFSPGGAISNMYSIMAARYKFFPEVKTKG
rat (from peptide)
YEIAPVFVLLEYV-----REIIGWPGGS-DGIFSPGGAISN-YAMLIARYKMFPEVKEKG

FIG. 1

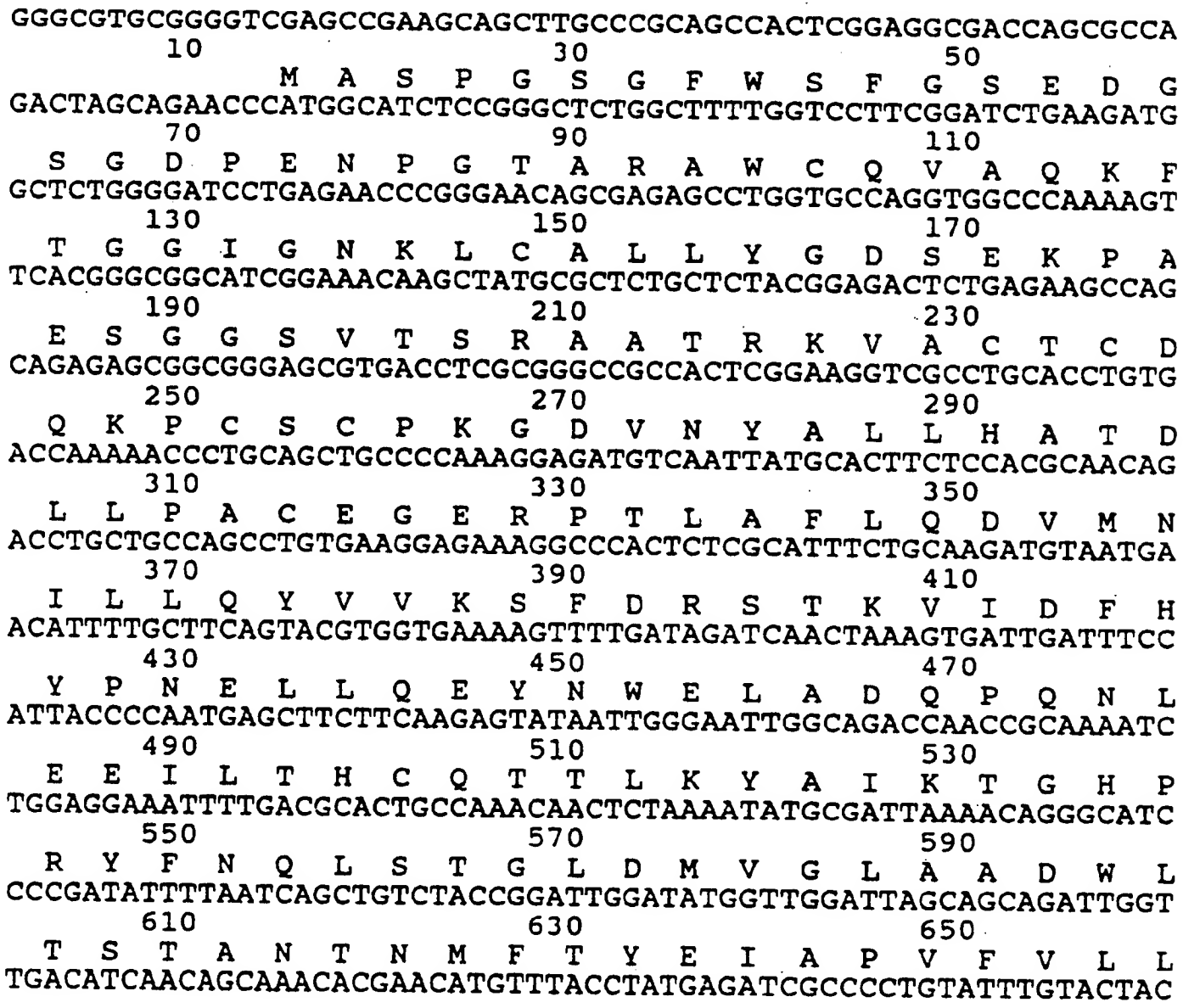


FIG. 2A

```
       670                       690                       710
  E  Y  V  T  L  K  K  M  R  E  I  I  G  W  P  G  G  S  G  D
TGGAATATGTGACACTAAAGAAAATGAGGGAAATCATTGGCTGGCCAGGAGGCTCTGGCG
       730                       750                       770
  G  I  F  S  P  G  G  A  I  S  N  M  Y  A  M  L  I  A  R  Y
ATGGAATCTTTTCTCCTGGTGGTGCCATCTCCAACATGTACGCCATGCTCATTGCCCGCT
       790                       810                       830
  K  M  F  P  E  V  K  E  K  G  M  A  A  V  P  R  L  I  A  F
ATAAGATGTTTCCAGAAGTCAAGGAAAAGGGGATGGCGGCGGTGCCCAGGCTCATCGCAT
       850                       870                       890
  T  S  E  H  S  H  F  S  L  K  K  G  A  A  A  L  G  I  G  T
TCACGTCAGAGCATAGTCACTTTTCTCTCAAGAAGGGAGCTGCAGCCTTGGGGATCGGAA
       910                       930                       950
  D  S  V  I  L  I  K  C  D  E  R  G  K  M  I  P  S  D  L  E
CAGACAGCGTGATTCTGATTAAATGTGATGAGAGAGGGAAAATGATCCCATCTGACCTTG
       970                       990                      1010
  R  R  I  L  E  V  K  Q  K  G  F  V  P  F  L  V  S  A  T  A
AAAGAAGAATCCTTGAAGTCAAACAGAAAGGATTTGTTCCTTTCCTGGTGAGTGCCACAG

  R  R  I  L  E  V  K  Q  K  G  F  V  P  F  L  V  S  A  T  A
AAAGAAGAATCCTTGAAGTCAAACAGAAAGGATTTGTTCCTTTCCTGGTGAGTGCCACAG
      1030                      1050                      1070
  G  T  T  V  Y  G  A  F  D  P  L  L  A  V  A  D  I  C  K  K
CTGGAACCACTGTGTACGGGGCTTTTGATCCTCTCTTGGCTGTAGCTGACATCTGCAAAA
      1090                      1110                      1130
  Y  K  I  W  M  H  V  D  A  A  W  G  G  G  L  L  M  S  R  K
AATATAAGATCTGGATGCATGTGGATGCTGCTTGGGGTGGAGGGTTACTGATGTCTCGGA
      1150                      1170                      1190
  H  K  W  K  L  N  G  V  E  R  A  N  S  V  T  W  N  P  H  K
AACACAAGTGGAAGCTGAACGGTGTGGAGAGGGCCAACTCTGTGACATGGAATCCCCACA
      1210                      1230                      1250
  M  M  G  V  P  L  Q  C  S  A  L  L  V  R  E  E  G  L  M  Q
AGATGATGGGTGTCCCCTTGCAATGTTCGGCTCTCCTGGTCAGAGAGGAGGGACTGATGC
```

FIG. 2B

```
     1270                1290                1310
  S  C  N  Q  M  H  A  S  Y  L  F  Q  Q  D  K  H  Y  D  L  S
AGAGCTGCAACCAGATGCATGCTTCCTACCTCTTTCAGCAAGATAAGCACTATGACCTGT
     1330                1350                1370
  Y  D  T  G  D  K  A  L  Q  C  G  R  H  V  D  V  F  K  L  W
CCTATGACACGGGAGACAAGGCCTTGCAGTGTGGACGCCACGTCGATGTCTTTAAATTAT
     1390                1410                1430
  L  M  W  R  A  K  G  T  T  G  F  E  A  H  I  D  K  C  L  E
GGCTCATGTGGAGAGCAAAGGGGACTACTGGATTTGAAGCTCACATTGATAAGTGTTTGG
     1450                1470                1490
  L  A  E  Y  L  Y  N  I  I  K  N  R  E  G  Y  E  M  V  F  D
AGCTGGCAGAGTATTTATACAATATCATTAAAAACCGAGAAGGATATGAAATGGTGTTCG
     1510                1530                1550
  G  K  P  Q  H  T  N  V  C  F  W  F  V  P  P  S  L  R  V  L
ATGGGAAGCCTCAGCACACAAATGTCTGCTTCTGGTTTGTACCTCCTAGTTTGCGAGTTC
     1570                1590                1610
  E  D  N  E  E  R  M  S  R  L  S  K  V  A  P  V  I  K  A  R
TGGAAGACAATGAAGAGAGAATGAGCCGCCTCTCAAAGGTGGCGCCAGTGATTAAAGCCA
     1630                1650                1670
  M  M  E  Y  G  T  T  M  V  S  Y  Q  P  L  G  D  K  V  N  F
GAATGATGGAGTATGGGACCACAATGGTCAGCTACCAACCCTTAGGAGATAAGGTCAACT
     1690                1710                1730
  F  R  M  V  I  S  N  P  A  A  T  H  Q  D  I  D  F  L  I  E
TCTTCCGCATGGTCATCTCAAACCCTGCAGCAACTCACCAAGACATTGACTTCCTCATTG
     1750                1770                1790
  E  I  E  R  L  G  Q  D  L  *
AAGAAATCGAACGCCTGGGACAAGATTTGTAATCACTTTGCTCACCAAACTTTCAGTTCT
     1810                1830                1850
CTAGGTAGACAGCTAAGTTGTCACAAACTGTGTAAATGTATTTGTAGTTTGTTCCAGAGT
     1870                1890                1910
AATTCTATTTCTATATCGTGGTGTCACAGTAGAGTCCAGTTTAAAA
     1930                1950
```

FIG. 2C

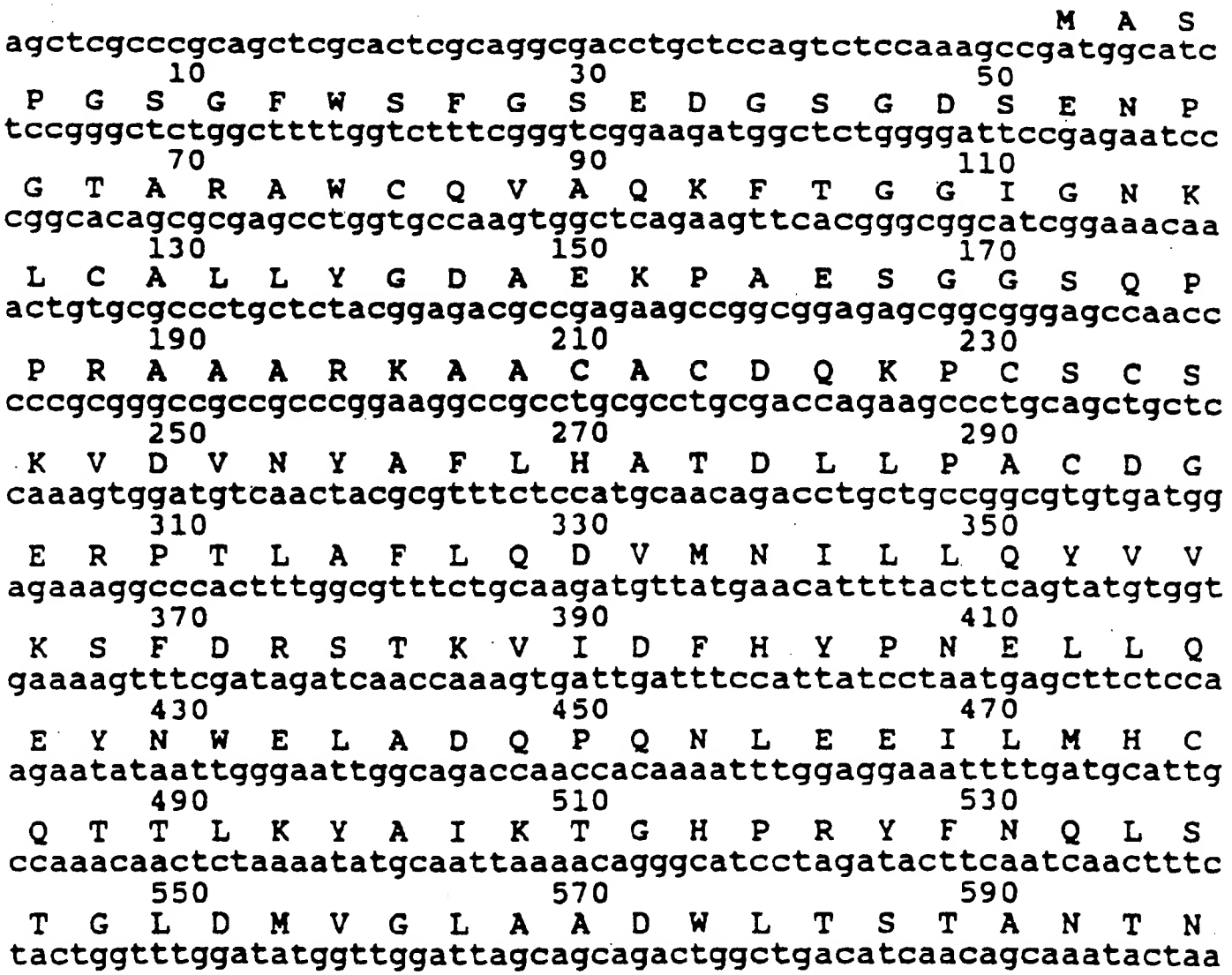


FIG. 3A

```
         610                             630                            650
 M  F  T  Y  E  I  A  P  V  F  V  L  L  E  Y  V  T  L  K  K
catgttcacctatgaaattgctccagtatttgtgcttttggaatatgtcacactaaagaa
         670                             690                            710
 M  R  E  I  I  G  W  P  G  G  S  G  D  G  I  F  S  P  G  G
aatgagagaaatcattggctggccaggggctctggcgatgggatatttctcccggtgg
         730                             750                            770
 A  I  S  N  M  Y  A  M  M  I  A  R  F  K  M  F  P  E  V  K
cgccatatctaacatgtatgccatgatgatcgcacgctttaagatgttcccagaagtcaa
         790                             810                            830
 E  K  G  M  A  A  L  P  R  L  I  A  F  T  S  E  H  S  H  F
ggagaaaggaatggctgctcttcccaggctcattgccttcacgtctgaacatagtcattt
         850                             870                            890
 S  L  K  K  G  A  A  A  L  G  I  G  T  D  S  V  I  L  I  K
ttctctcaagaagggagctgcagccttaggattggaacagacagcgtgattctgattaa
         910                             930                            950
 C  D  E  R  G  K  M  I  P  S  D  L  E  R  R  I  L  E  A  K
atgtgatgagagagggaaaatgattccatctgatcttgaaagaaggattcttgaagccaa
         970                             990                           1010
 Q  K  G  F  V  P  F  L  V  S  A  T  A  G  T  T  V  Y  G  A
acagaaagggtttgttcctttcctcgtgagtgccacagctggaaccaccgtgtacggagc
        1030                            1050                           1070
 F  D  P  L  L  A  V  A  D  I  C  K  K  Y  K  I  W  M  H  V
atttgacccccctcttagctgtcgctgacatttgcaaaaagtataagatctggatgcatgt
        1090                            1110                           1130
 D  A  A  W  G  G  G  L  L  M  S  R  K  H  K  W  K  L  S  G
ggatgcagcttggggtgggggattactgatgtcccgaaaacacaagtggaaactgagtgg
        1150                            1170                           1190
 V  E  R  A  N  S  V  T  W  N  P  H  K  M  M  G  V  P  L  Q
cgtggagagggccaactctgtgacgtggaatccacacaagatgatgggagtccctttgca
```

FIG. 3B

```
     1210                          1230                          1250
C  S  A  L  L  V  R  E  E  G  L  M  Q  N  C  N  Q  M  H  A
gtgctctgctctcctggttagagaagagggattgatgcagaattgcaaccaaatgcatgc
     1270                          1290                          1310
S  Y  L  F  Q  Q  D  K  H  Y  D  L  S  Y  D  T  G  D  K  A
ctcctacctctttcagcaagataaacattatgacctgtcctatgacactggagacaaggc
     1330                          1350                          1370
L  Q  C  G  R  H  V  D  V  F  K  L  W  L  M  W  R  A  K  G
cttacagtgcggacgccacgttgatgtttttaaactatggctgatgtggagggcaaaggg
     1390                          1410                          1430
T  T  G  F  E  A  H  V  D  K  C  L  E  L  A  E  Y  L  Y  N
gactaccgggtttgaagcgcatgttgataaatgtttggagttggcagagtatttatacaa
     1450                          1470                          1490
I  I  K  N  R  E  G  Y  E  M  V  F  D  G  K  P  Q  H  T  N
catcataaaaaaccgagaaggatatgagatggtgtttgatgggaagcctcagcacacaaa
```

FIG. 3C

```
       1510                          1530                          1550
 V   C   F   W   Y   I   P   P   S   L   R   T   L   E   D   N   E   E   R   M
tgtctgcttctggtacattcctccaagcttgcgtactctggaagacaatgaagagagaat
       1570                          1590                          1610
 S   R   L   S   K   V   A   P   V   I   K   A   R   M   M   E   Y   G   T   T
gagtcgcctctcgaaggtggctccagtgattaaagccagaatgatggagtatggaaccac
       1630                          1650                          1670
 M   V   S   Y   Q   P   L   G   D   K   V   N   F   F   R   M   V   I   S   N
aatggtcagctaccaacccttgggagacaaggtcaatttcttccgcatggtcatctcaaa
       1690                          1710                          1730
 P   A   A   T   H   Q   D   I   D   F   L   I   E   E   I   E   R   L   G   Q
cccagcggcaactcaccaagacattgacttcctgattgaagaaatagaacgccttggaca
       1750                          1770                          1790
 D   L   *
agatttataataaccttgctcaccaagctgttccacttctctaggtagacaattaagttg
       1810                          1830                          1850
tcacaaactgtgtgaatgtatttgtagtttgttccaaagtaaatctatttctatattgtg
       1870                          1890                          1910
gtgtcaaagtagagtttaaaaattaaacaaaaaagacattgctccttttaaaagtccttt
       1930                          1950                          1970
cttaagtttagaatacctctctaagaattcgtgacaaaggctatgttctaatcaataag
       1990                          2010                          2030
gaaaagcttaaaattgttataaatacttcccttactttaatatagtgtgcaaagcaaac
       2050                          2070                          2090
```

FIG. 3D

GAP WEIGHT:	3.000	AVERAGE MATCH:	0.540
LENGTH WEIGHT:	0.100	AVERAGE MISMATCH:	-0.396
QUALITY:	856.2	LENGTH:	585
RATIO:	1.464	GAPS:	0
PERCENT SIMILARITY:	97.436	PERCENT IDENTITY:	96.068

4817, PEP HGT2.PEP AUGUST 22, 1990 08:20 ..

```
   1 MASPGSGFWSFGSEDGSGDPENPGTARAWCQVAQKFTGGIGNKLCALLYG  50
     ||||||||||||||||||||.||||||||||||||||||||||||||||
   1 MASPGSGFWSFGSEDGSGDSENPGTARAWCQVAQKFTGGIGNKLCALLYG  50

  51 DSEKPAESGGSVTSRAATRKVACTCDQKPCSCPKGDVNYALLHATDLLPA 100
     |.|||||||||| ..|||.||.||.|||||||||.|.|.|||:|||||||||
  51 DAEKPAESGGSQPPRAAARKAACACDQKPCSCSKVDVNYAFLHATDLLPA 100

 101 CEGERPTLAFLQDVMNILLQYVVKSFDRSTKVIDFHYPNELLQEYNWELA 150
     |:||||||||||||||||||||||||||||||||||||||||||||||||||
 101 CDGERPTLAFLQDVMNILLQYVVKSFDRSTKVIDFHYPNELLQEYNWELA 150

 151 DQPQNLEEILTHCQTTLKYAIKTGHPRYFNQLSTGLDMVGLAADWLTSTA 200
     |||||||||| ||||||||||||||||||||||||||||||||||||||||
 151 DQPQNLEEILMHCQTTLKYAIKTGHPRYFNQLSTGLDMVGLAADWLTSTA 200

 201 NTNMFTYEIAPVFVLLEYVTLKKMREIIGWPGGSGDGIFSPGGAISNMYA 250
     ||||||||||||||||||||||||||||||||||||||||||||||||||||
 201 NTNMFTYEIAPVFVLLEYVTLKKMREIIGWPGGSGDGIFSPGGAISNMYA 250

 251 MLIARYKMFPEVKEKGMAAVPRLIAFTSEHSHFSLKKGAAALGIGTDSVI 300
     |:|||:|||||||||||||:||||||||||||||||||||||||||||||||
 251 MMIARFKMFPEVKEKGMAALPRLIAFTSEHSHFSLKKGAAALGIGTDSVI 300

 301 LIKCDERGKMIPSDLERRILEVKQKGFVPFLVSATAGTTVYGAFDPLLAV 350
     ||||||||||||||||||||.||||||||||||||||||||||||||||||
 301 LIKCDERGKMIPSDLERRILEAKQKGFVPFLVSATAGTTVYGAFDPLLAV 350

 351 ADICKKYKIWMHVDAAWGGGLLMSRKHKWKLNGVERANSVTWNPHKMMGV 400
     ||||||||||||||||||||||||||||||.|||||||||||||||||||||
 351 ADICKKYKIWMHVDAAWGGGLLMSRKHKWKLSGVERANSVTWNPHKMMGV 400
```

FIG.4A

```
401 PLQCSALLVREEGLMQSCNQMHASYLFQQDKHYDLSYDTGDKALQCGRHV 450
    ||||||||||||||||||.||||||||||||||||||||||||||||||||
401 PLQCSALLVREEGLMQNCNQMHASYLFQQDKHYDLSYDTGDKALQCGRHV 450

451 DVFKLWLMWRAKGTTGFEAHIDKCLELAEYLYNIIKNREGYEMVFDGKPQ 500
    ||||||||||||||||||||||:||||||||||||||||||||||||||||
451 DVFKLWLMWRAKGTTGFEAHVDKCLELAEYLYNIIKNREGYEMVFDGKPQ 500

501 HTNVCFWFVPPSLRVLEDNEERMSRLSKVAPVIKARMMEYGTTMVSYQPL 550
    |||||||:::||||||.||||||||||||||||||||||||||||||||||
501 HTNVCFWYIPPSLRTLEDNEERMSRLSKVAPVIKARMMEYGTTMVSYQPL 550

551 GDKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL 585
    ||||||||||||||||||||||||||||||||||||
551 GDKVNFFRMVISNPAATHQDIDFLIEEIERLGQDL 585
```

FIG.4B

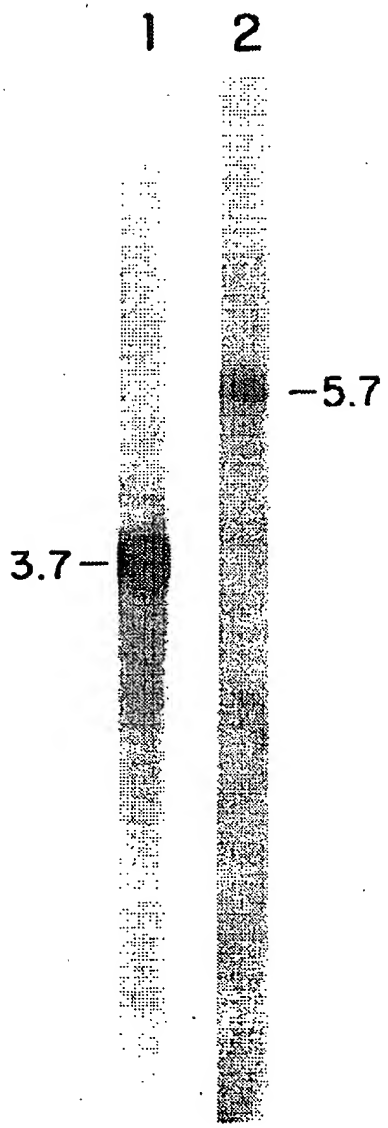


FIG.5

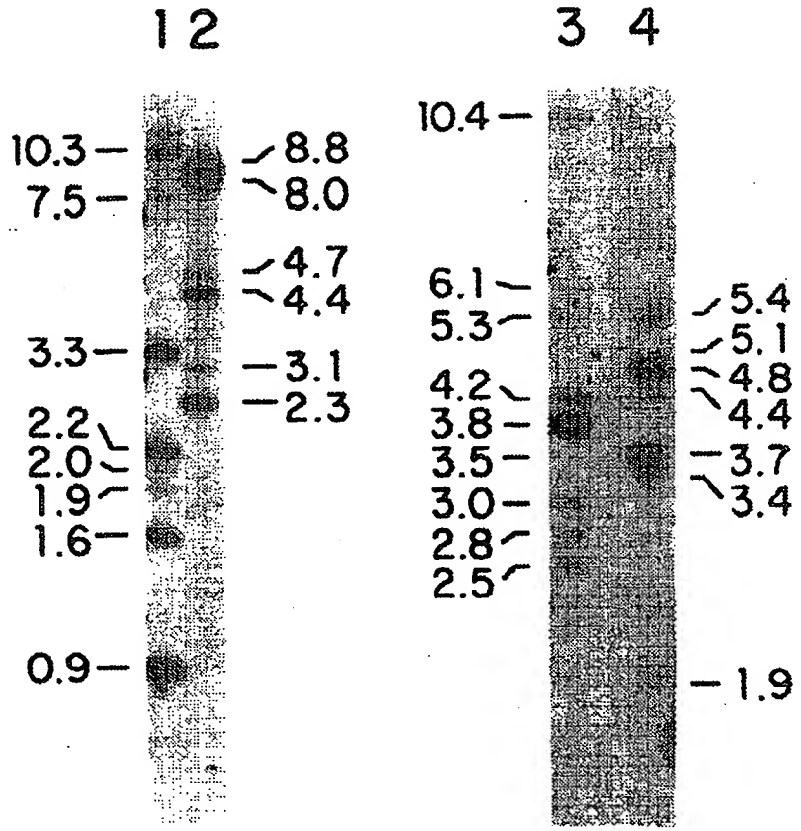

1 2
3 4
10.3
7.5
3.3
2.2
2.0
1.9
1.6
0.9
8.8
8.0
4.7
4.4
3.1
2.3
10.4
6.1
5.3
4.2
3.8
3.5
3.0
2.8
2.5
5.4
5.1
4.8
4.4
3.7
3.4
1.9

FIG. 6

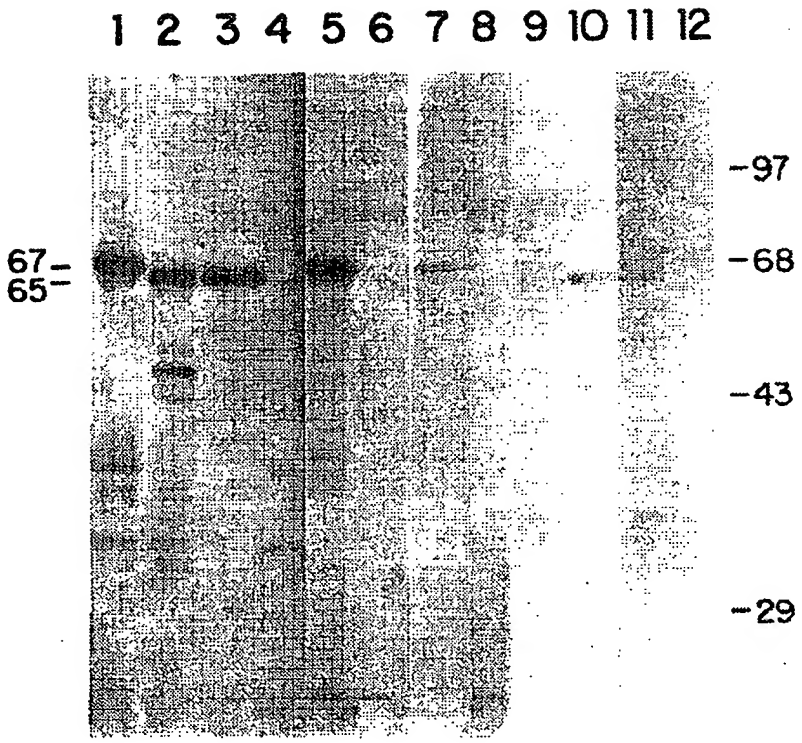


FIG.7

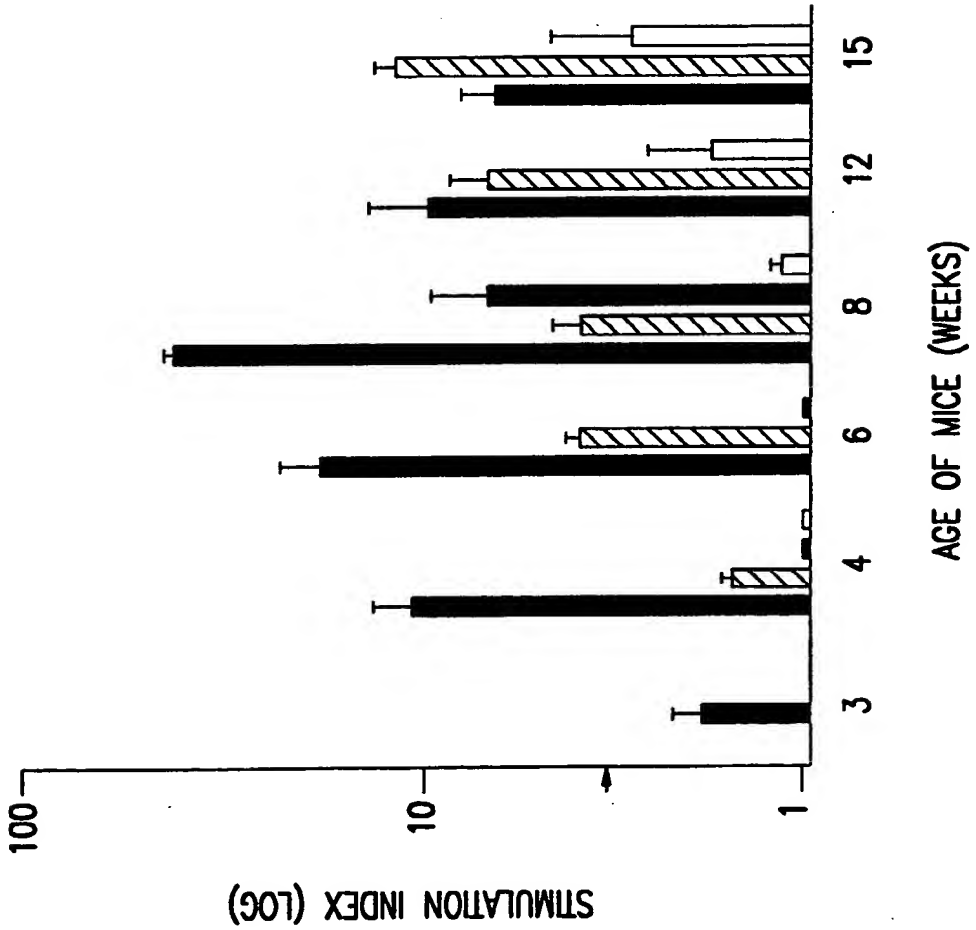


FIG.8

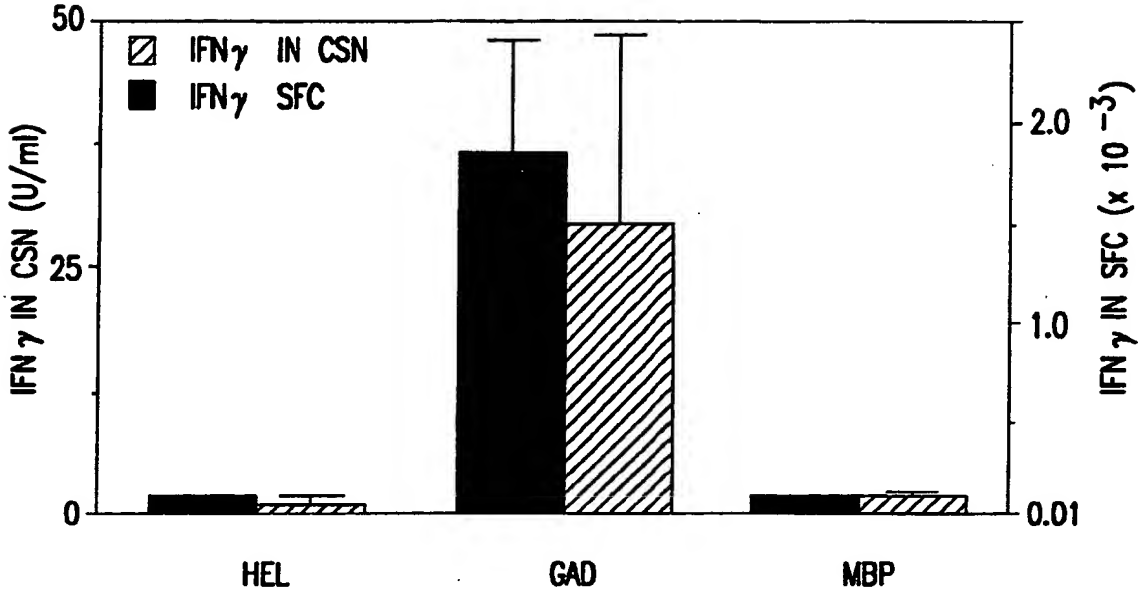

IFNγ IN CSN
IFNγ SFC
IFN γ IN CSN (U/ml)
IFN γ IN SFC (x 10 -3)
50
25
0
2.0
1.0
0.01
HEL
GAD
MBP

FIG.9A

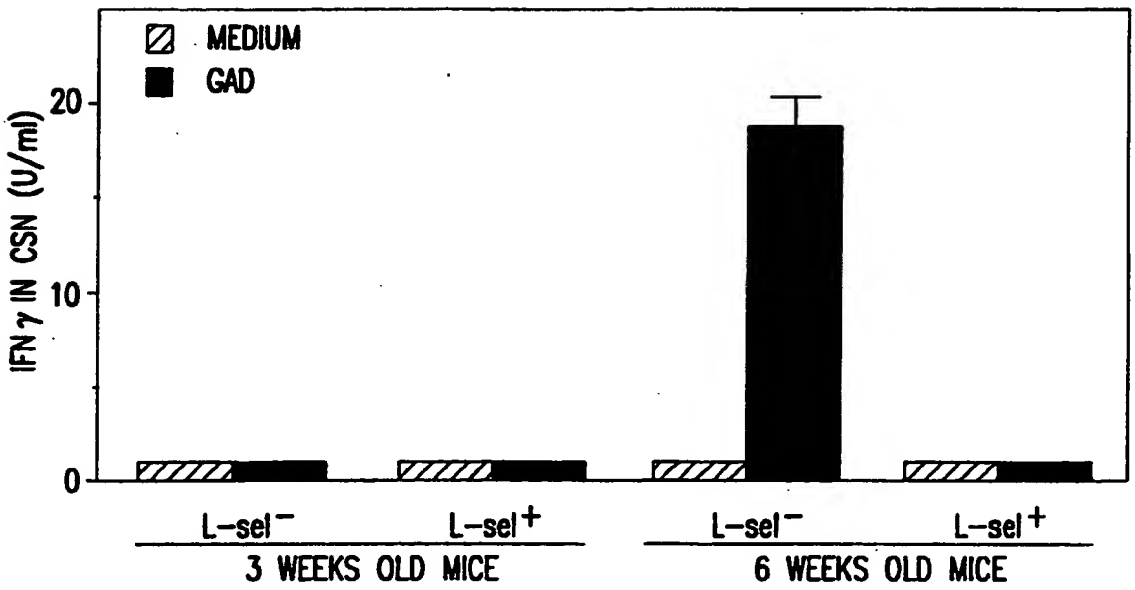

MEDIUM
GAD
IFN γ IN CSN (U/ml)
20
10
0
L-sel-
L-sel+
L-sel-
L-sel+
3 WEEKS OLD MICE
6 WEEKS OLD MICE

FIG.9B

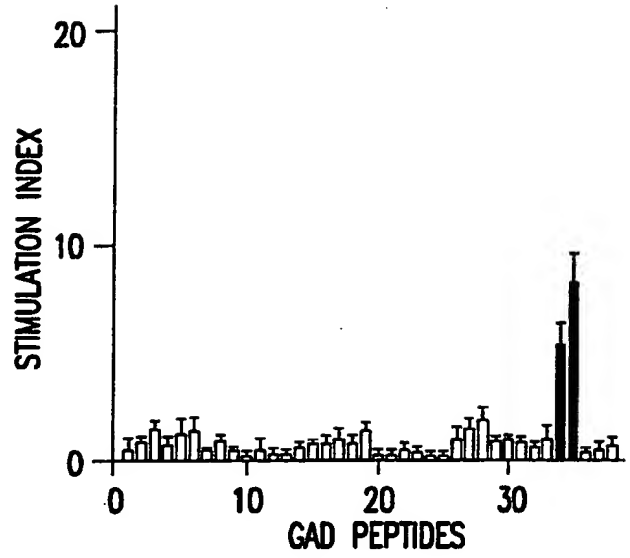


FIG.10A

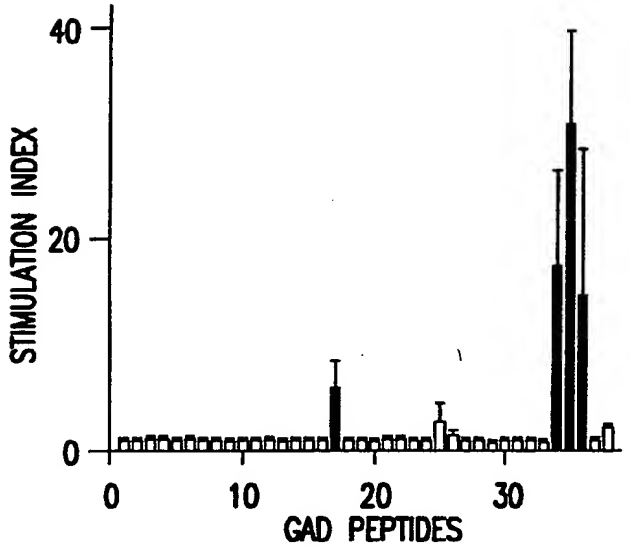


FIG.10B

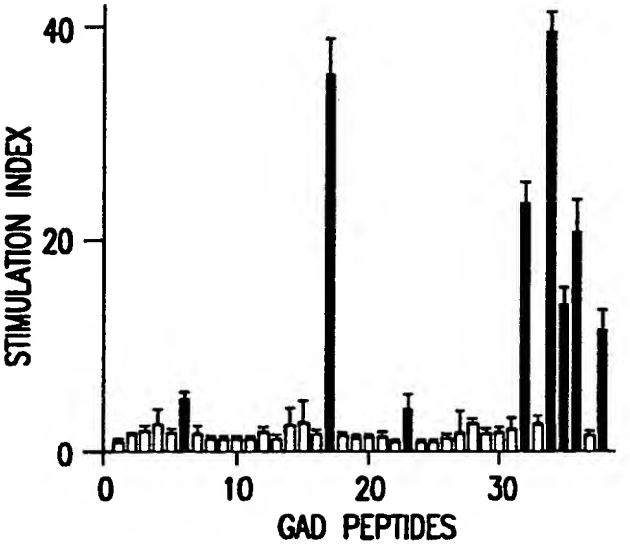
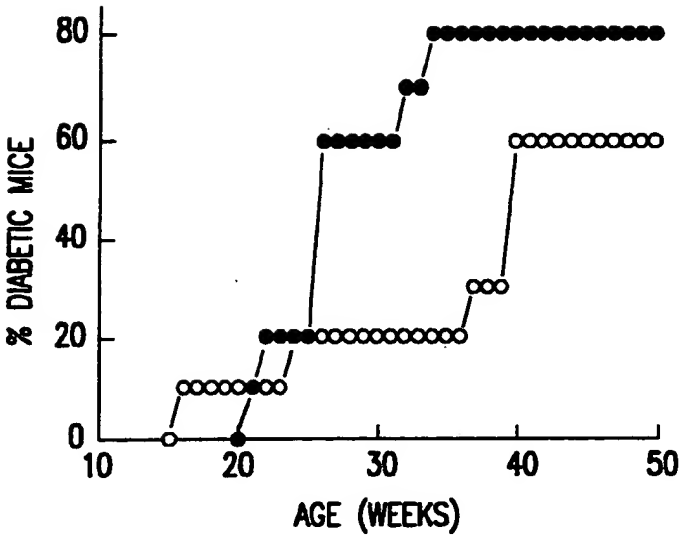


FIG.10C



o = MICE RECEIVING GAD IN IFA

● = MICE RECEIVING CONTROL β -GAL IN IFA

FIG.11